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13. ABSTRACT (Maximum 200 Words) Mechanical stimulation is crucial to the homeostasis of adult bone density and mass. The hypothesis of this proposal is that bone cells sense their mechanical environment through specific cell surface receptors (integrins) that interact with specific extracellular matrix (ECM) proteins (osteopontin, bone sialoprotein, and fibronectin) that are the ligands for these receptors. We propose that the expression of these proteins is regulated in response to both cellular interactions with the ECM and mechanical stimulation. Thus, these proteins act like autocrine factors that modify cell behavior in response to changes in either matrix composition or mechanical deformation of the ECM itself. The proposed experiments will define how osteoblasts discriminate the molecular mechanisms by which mechanical signals mediat thier actions through the cellular interactions of integrins with the ECM. A determination of the specific integrin isotypes that are involved in the mechano-signal transduction process will be made. The signal transduction system(s) that are responsible for mediating osteopontin, bone sialoprotein and fibronectin gene expression in response to mechanical stimulation, will be determined. Other experiments will examine how aspects of the mechanical stimuli, such as frequency, intensity or duration effect cell response. Knowledge gained from understanding mechano-signal transduction will facilitate the development of appropriate clinical approaches to enhance the adaptive responses of skeletal tissue to mechanical stimulation.

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FOREWORD

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86-23, Revised 1985).

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Biosafety in Microbiological and Biomedical Laboratories.

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INTRODUCTION

The bone remodeling cycle is known to be intimately involved in the metabolic homeostasis of mineral balance.[1] Bone formation and the remodeling cycle has been shown to be essential in maintaining the structural integrity of skeletal tissue in response to the mechanical loading to which it is subjected.[2,3] Bone remodeling has also been hypothesized to provide the means of repairing bone tissue that has been damaged as a result of mechanical fatigue.[4] Thus, it may speculated that the skeletal cells (osteoblasts and osteoclasts) which mediate the remodeling process are regulated by their mechanical environment. In order for osteoblasts to respond to their mechanical environment, they must in some way sense it. One mechanism cells might sense mechanical signals is through the physical deformation of the tissue. The hypothesis of this proposal is that specific cell surface receptors (integrins) that interact with specific extracellular matrix proteins (osteopontin, bone sialoprotein, and fibronectin) provide the physical link through which mechanical stimuli is transmitted via tissue deformation. We further hypothesis that these extracellular protein that are ligands for these receptors are regulated in response to both cellular interaction with the matrix and mechanical stimulation. Thus, these proteins act both like autocrine factors that modify cell behavior in response to changes in matrix composition and mechanical deformation as well as having a structural role in the matrix itself. It is through the maintenance of the balance of the composition of these various proteins in the extracellular matrix, that matrix homeostasis in response to its structural needs is then maintained. **A review of the major issues of mechano-signal transduction, as it relates to osteopontin expression is provided in appendix 1.**

The proposed experiments are designed to define how osteoblasts discriminate at a molecular level both mechanical signals and extracellular matrix mediated signals, that are transduced through integrin receptor. They will define whether different genes use common mechanisms in the regulation of their response to these stimuli. A determination of the Extracellular Receptor Kinase (ERK) system(s) that are responsible for mediating the altered gene expression of opn, bsp and fn to mechanical stimulation will be determined, both by assessing which ERK systems are activated and through the use of specific inhibitors that block the actions of the different ERK systems. A determination will be made if specific integrin isotypes are differentially involved in the different signal transduction processes. Other experiments will examine if individual gene responses are differentially sensitive to various aspects of the mechanical stimuli (intensity, frequency, duration) or have different thresholds of response to different component parts of the stimuli. Knowledge gained from understanding mechano-signal transduction will facilitate the development of appropriate clinical approaches to enhance the adaptive responses of skeletal tissue to mechanical stimulation.

BODY

Goal 1. The first goal of this proposal is directed at defining the molecular mechanisms of signal transduction by which mechanical stimulation regulates the expression of three specific ECM genes (osteopontin, bone sialoprotein and fibronectin) within osteoblasts. These studies will test whether there are common signal transduction pathways that mediate changes in the expression of these genes in response to mechanical stimulation.

a) The involvement of specific integrin isotypes in the mechano-signal transduction process that mediates these gene's genomic responses to mechanical stimulation will be assessed. Year 1

b) The mechanistic relationship between the signal transduction pathways that are activated by mechanical stimulation vs. cell adhesion will be examined. Years 1-3
c) The nature of the second signal transduction pathways that mediate the changes in expression for each of these genes will be determined. Years 3-4

Towards the completion of goal 1 we have carried out two sets of studies. In the first study we define the variations in signal transduction mechanisms that were used to stimulate the expression of three separate RGD containing proteins in osteoblasts. This study examined if osteoblast expression of other integrin binding proteins such as fibronectin *(fn)* and bone sialoprotein *(bsp)* would be similarly responsive to mechanical stimulation. All three genes showed 3 to 4 fold maximal induction in response to a single two hour period of an applied dynamic spatially uniform biaxial strain of 1.3% strain at 0.25 Hz. Each gene however had a different time course of induction with *bsp fn* and *opn* showing their maximal response at one three and nine hours respectively after the stimulation period. In contrast the levels of both collagen type I and osteocalcin mRNAs were unaltered over the same time course of examination. The effect of cell adhesion on the expression of these genes was separately examined to determine if this stimuli would mimic the effects of the mechanical stimulation. All three genes also showed comparable levels of induction in response to adhesion on the fibronectin coated surfaces in comparison to non-coated surfaces with maximal levels of induction seen for *bsp* and *opn* at 24 hours after plating while *fn* showed maximal levels of stimulation at 8 hours. Interestingly while both *opn* and *fn* mRNA expression returned to base line after cell adhesion on fibronectin *bsp* mRNA levels remained elevated. Examination of the signal transduction pathways that mediated the gene expression in response to attachment on fibronectin coated surfaces showed that both genistein and cycloheximide inhibited the induction of all three genes demonstrating that a tyrosine kinase was involved in the cell attachment mediated induction of these genes and new protein synthesis was a prerequisite to this process. In contrast the PKA specific inhibitor H-89 only ablated the induction of *fn* expression. Depolymerization of either microtubules or microfilaments with colchicine or cytochalasin D respectively had little effect on the over all expression of these gene in response to cell adhesion indicating that the adhesion mediated phenomom was not dependnet on cytoskeletal integrity. In summary, these results show that both mechanical stimulation and cell adhesion specifically stimulated the expression of integrin binding proteins. These results further demonstrate that while there are common features in the signal transduction processes that mediated the induction of these genes, each gene was separately induced by unique mechanisms. **A complete description of these finding are in appendix two.**

In the second study we examined if cell adhesion to various integrin ligands would mimic the processes of mechano-transduction that stimulated *opn* mRNA expression. These studies also examined if integrin interactions with specific ligands were involved in these processes. Osteoblast adhesion was selectively mediated when embryonic chicken calvaria osteoblasts were plated on fibronectin (FN), native collagen type I, denatured collagen type I (gelatin) and laminin coated tissue culture surfaces. Osteopontin and vitronectin did not facilitate specific cell adhesion. We then measured the induction of osteopontin expression at 24 hours after the cells were plated on tissue culture plastic alone or coated with these various extracellular matrix molecules. The strongest induction was seen with fibronectin, native collagen and laminin while denatured collagen was weakly inducing and neither osteopontin or vitronectin was capable of

A. SPECIFIC ATTACHMENT OF OSTEOBLASTS
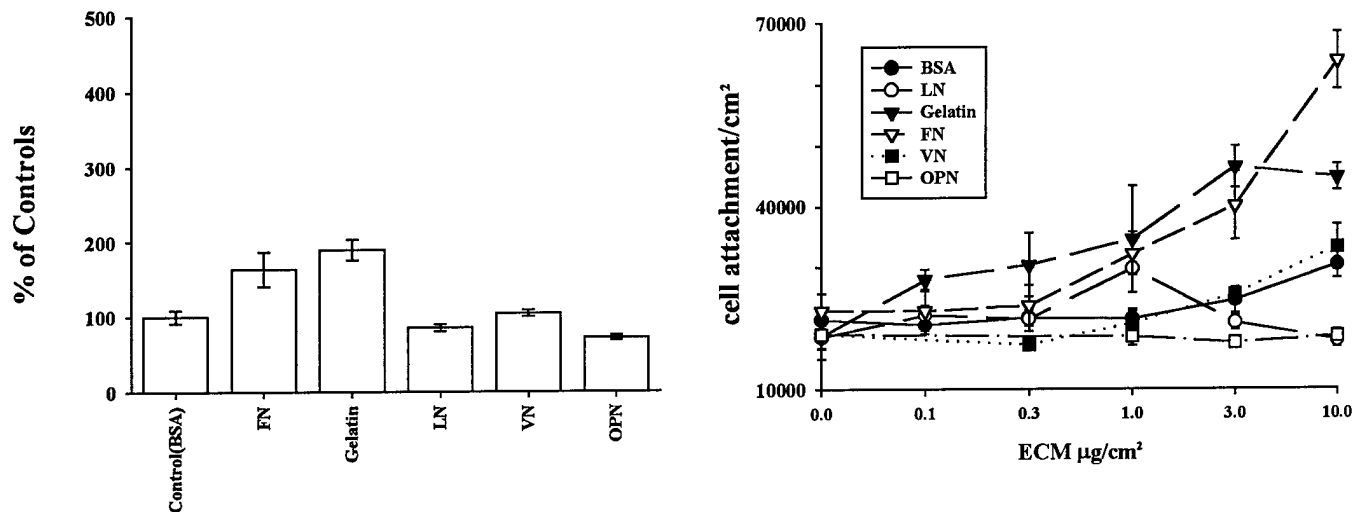
B. INDUCTION OF OPN mRNA
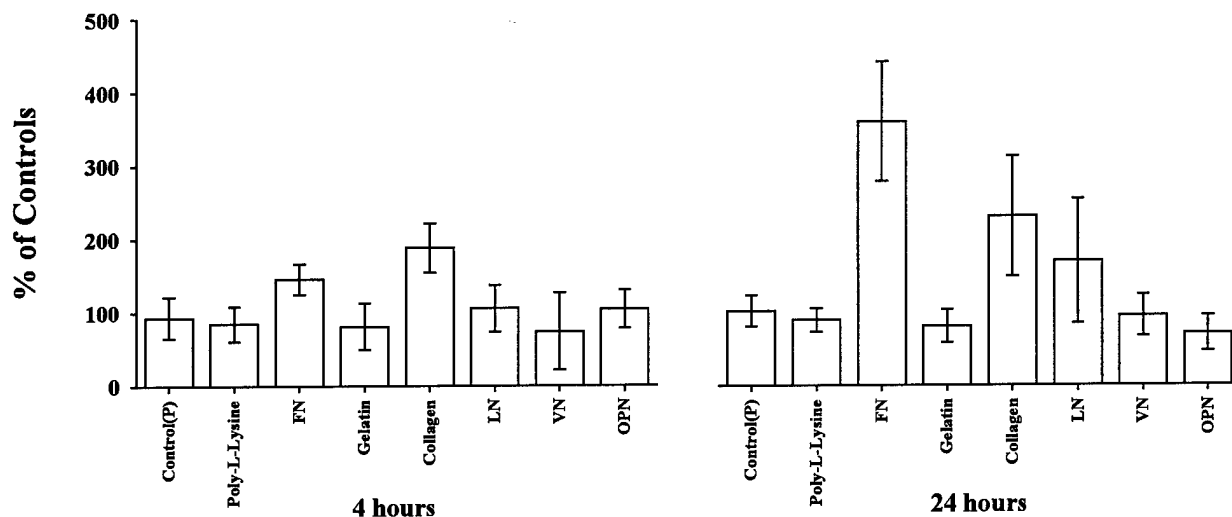
Figure 1 Comparison of selective attachment with induction of osteopontin in response to cell plating on different integrin ligands.   Panel A   Selective attachment of 17 day embryonic chick osteoblasts on different ligands after a four hour period.   Left panel depicts the percent attachment compared to control on poly l-lysine coated surfaces after hours.   All protein concentrations were at $2\mu g/cm^2$.   Right panel depicts the total number of cells that were attached after four hours as a function of varying the concentration of the ligands.

Panel B.   Induction of osteopontin expression in response to cell attachment on various ECM proteins. Osteopontin expression was measured by Northern blot analysis and the band intensities were normalized to the 18s band(data not shown).   Left panel shows percent induction after four hours and right panel after 24 hours.   The nature of the protein coatings are denoted in the figure

inducing osteopontin mRNA expression. These results are seen in Figure one. These results suggest that the same integrin receptors that facilitate specific cell attachment also facilitate the induction of osteopontin expression. It is interesting to note that neither osteopontin not vitronectin mediate either of these cell responses suggesting that they are not facilitated through a $\alpha_v\beta_3$ receptor. The one differences that was observed however was in the comparison of cell attachment on denatured collagen vs. the induction of osteopontin. This result may suggest that signal transduction through the collagen receptor is only mediated when it interacts with native collagen. Concurrent with this study, we collected cells that were plated on the various ECM molecules and that were mechanically stimulated. Intracellular membrane associated and cytosolic proteins were then isolated. These protein fractions are currently being assayed for the induction of various kinases by Dr. Salih.

Goal 2 The second goal of these studies will determine the relative importance of each component
part of the mechanical stimuli (intensity, frequency and duration) to the mechano-signal transduction process.
  a) The component of the mechanical stimuli (intensity, frequency, duration) that is responsible for producing the signal transduction which leads to the genomic regulation of specific genes in osteoblasts will be examined. Years 1-2
  b)   Short term (<24hr) vs. chronic (>72hr) adaptive responses of osteoblasts to variations in duration of mechanical perturbation will be defined. Years 3-4

       Towards the completion of goal two, initial studies were carried out to determine the component part of mechanical stimuli that would induce changes in osteopontin gene expression. In these studies we analyzed duration and frequency of the mechanical stimulation and examined the effect of fluid flow-induced shear stress on osteopontin expression. Using our device that delivers spatially uniform biaxial strain to a membrane surface, osteoblasts were subjected to 1% strain for 1,5,15,30 minutes and 1,2,4,and 8 hours of strain. Analysis of the induction of osteopontin mRNA expression demonstrated that a maximal 145% induction was observed after 8 hours. ($p.<0.05$). Osteoblasts were then subjected to varying frequencies of strain (0.1, 0.25, 0.5 and 1 Hz) for 8 hours. There was clearly a 2-3 fold stimulation in response to increasing frequency again with a significant finding ($p<0.05$). The effect of fluid induced shear stress was examined in the third part of this study. This was accomplished in the following manner. The membrane was cut into two equal areas that encompassed the inner and outer circular areas of the membrane. The cells were then subjected to an 8 hour period of mechanical stimulation at the same variations in frequency as used in the first part of these studies. Because of the way the device generates its strains the inner circular area has several fold lower fluid flow than the outer areas. Similarly higher frequencies will generate higher fluid flows thus the combination of inner and outer areas with increasing frequency of strain should produce several orders of magnitude greater fluid movement. Analysis of osteopontin mRNA production, however, showed that only the highest frequency (1 Hz) generated altered levels of osteopontin expression when comparing the inner and outer areas of the membrane. These results also showed that it was inversely related to the levels of fluid flow. Thus, the inner areas had the higher levels of osteopontin induction. These results were completely opposite to the hypothesis that fluid shear stress was

the major mechanical stimulation that facilitated osteopontin expression. These results also would lead to questions as to whether Ca flux through stress activated channels was a component part of the signal transduction mechanisms that induced osteopontin expression as has been suggested by other research groups.[5] **A complete description of these finding are in appendix three.**

KEY RESEARCH ACCOMPLISHMENTS

1. We have completed one the (a) component of goal one.

2. We have partially completed the (b) component of goal one.

3. Experiments were initiated for the (c) component of goal one and are ongoing.

4. We have partially completed the (a) component of goal two

5. We have not as yet begun the experiments as outlined in the (b) component of goal two.

REPORTABLE OUTCOMES

Manuscripts, abstracts ,presentations

Manuscripts
a)Gerstenfeld, LC. Editorial: Research Procpective J. Bone and Mienral Research 1999 14:850-855 Osteoponetin in skeletal tissue homestasis: an emerging picture of the autocrine/parcrine functions of the extracellular matrix

b) Carvalho, RS, Schaffer, JL, Bumann, A, and Gerstenfeld LC. Integrin ligands expressed by osteoblasts show preferential regulation in response to both cell adhesion and mechanical stimulation (Unpublished manuscript submitted 1999, Bone)

Absrtacts
a) Differential Mechanisms of Signal Transduction that Mediate the Induction of Gene Expression by Cell Adhesion or Mechanical Stimulation within Osteoblasts Roberto S. Carvalho, Paul Kostenuick, Axel Bumann, and Louis C. Gerstenfeld

Presentations
a)Differential Mechanisms of Signal Transduction that Mediate the Induction of Gene Expression by Cell Adhesion or Mechanical Stimulation within Osteoblasts
At Osteoporosis as a failure of Bone's adaptation to functional load bearing The Wellecome Trust Foundation, Highgate, England

Patents None

Degrees obtained that are supported by this award
Samuel, E J. Masters Thesis Harvard School of Dental Medicine, Title of thesis "Osteoblast induction of osteopontin expression: response to changes in duration and frequency of mechanical strain, and to variation in flow induced shear stress"

Development of cell lines, tissue or serum repositories; None

Informatics such as databases and animal models, etc; None

Funding applied for based on work supported by this award; None
Employment or research opportunities applied for and/or received on experiences/training
supported by this award.None

CONCLUSIONS

The major conclusions from the first year of funding of this proposal are as follows:

1. Integrin ligands as a class are induced in osteoblasts in response to mechanical stimulation
and adhesion. Such finding provides strong evidence to support the hypothesis that these
molecules act like autocrine or paracrine factors.

2. Cell adhesion of osteoblasts is specifically mediated by $\beta 1$ class of integrins. This same class
of integrins appears to be responsible for the signal transduction process that stimulates
osteopontin induction. The $\beta 3$ integrin ligands vitronectin and osteopontin neither mediate
specific adhesion or induce osteopontin gene expression.

3. Component aspects of the mechanical stimulation do effect the induction of the gene. Both
duration and increasing frequency clearly appears to increase response to the mechanical signal.
Shear stress induced by fluid flow does not appear to be a mediating factor in osteopontin
induction in response to mechanical stimulation.

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14:103-109.

5 Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE, Duncan RL 1997 Mechanotransduction
in bone: osteoblasts are more responsive to fluid forces than mechanical strain . Am J Physiol
;273:C810-

APPENDICES
a) Gerstenfeld, LC. Editorial: Research Procpective J. Bone and Mienral Research 1999 14:850-
855 Osteoponetin in skeletal tissue homeostasis: an emerging picture of the autocrine/parcrine
functions of the extracellular matrix

b) Carvalho, RS, Schaffer, JL, Bumann, A, and Gerstenfeld LC. Integrin ligands expressed by osteoblasts show preferential regulation in response to both cell adhesion and mechanical stimulation (Unpublished manuscript will be submitted 1999, Bone)

c) Samuel, E J. Osteoblast induction of osteopontin expression: respponse to changes in duration and frequency of mechanical strain, and to variation in flow induced shear stress Masters Thesis Harvard School of Dental Medicine

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Louis C. Gerstenfeld PhD
Associate Professor
Orthopedic Surgery
Boston University Medical Center
715 Albany Street, Housman 2
Boston   MA 02118

8/5/99

RE: Application No.   98-096   Agency: Department of the Army

Title: Mechanisms of Mechano-Transduction Within Osteoblasts

Protocol Status: APPROVED,   7/22/98
ANIMAL NUMBERS/YR.   1248 chicken embryos/year x 4 yrs

BIOHAZARDS:

Dear Dr. Gerstenfeld
        Your application for use of animals in research or education has been reviewed by the Institutional Animal Care
and Use Committee at Boston University Medical Center. The protocol is APPROVED as being consistent with humane
treatment of laboratory animals and with standards set forth in the Guide for the Care and Use of Laboratory Animals and the
Animal Welfare Act.
        The Laboratory Animal Science Center at Boston University Medical Center has been accredited by the American
Association for Accreditation of Laboratory Animal Care since 1971. Boston University Medical Center has had an Animal
Welfare Assurance on file with the Office for Protection from Research Risks (OPRR) since January 1, 1986. Boston
University's Animal Welfare Assurance number is A-3316-01.
        . Animal protocols may be approved for up to three years. However, if the study extends beyond
one year from the approval date, an annual continuation form (1 page) must be submitted. If a project is to
extend beyond three years, a full application must be resubmitted and reviewed at the end of the initial three
year period.

Sincerely,

Colleen A. Cody, Coordinator
Institutional Animal Care and Use Committee

c: Wayne W. LaMorte, M.D., Ph.D., M.P.H. Chairman, IACUC
    Veterinary Staff, LASC

BOSTON UNIVERSITY MEDICAL CENTER

Radiation Protection
Office

88 East Newton Street, D-604
Boston, Massachusetts
02118-2394
617 638-7052

To: Dr. Gerstenfeld

Date: June 24, 1998

From: Victor Evdokimoff, Secretary Radioisotope Committee *VNE/Ch*

Subject: Authorization to use radioisotopes at BUMC

On June 24, 1998 your application __X__, renewal ____, amendment____ to use radioisotopes at BUMC was approved. You are only authorized for the following isotope(s), quantities, etc.

Isotope(s)	Form	Max./order	Max./year	Possession limit
H-3	Amino acid, Nucleotide	5 mCi	N/A	N/A
P-32	Phosphorous, Nucleotide	5 mCi	N/A	N/A
C-14	Chloramphenicol, Amino acids	1 mCi	N/A	N/A
S-35	Na Sulfate, Nucleotide, Amino acids	1 mCi	N/A	N/A
P-33	Phosphorous, Nucleotides	1 mCi	N/A	N/A

Your authorization code number is __G-16__. This number must appear on all requisitions for isotopes. In addition, the following **conditions** apply to your authorization:

1) The Radioisotope Committee is recommending you consider alternatives to using 3H/14C such as non-radioactive tracers.

2) All personnel under your permit planning on using P-32 must complete the individual training requirements. In addition, any user of 1 mCi or more of P-32 at one time is required to be monitored by TLD. Please contact the RPO for assistance.

Boston University
Medical Campus

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Administration

715 Albany Street, 560
Boston, Massachusetts
02118-2394
617 638-4600

BOSTON UNIVERSITY MEDICAL CENTER

Institutional Biosafety Committee

Renewal Letter: Biohazard Project

IBC Coordinator: Mary Gistis

Principal Investigator: Dr. Louis Gerstenfeld

Project Title: Musculoskeletal Research

Approval Number: # A-168

Renewal Date: November 4, 1998

Containment Level: BL-2/Universal Precautions

Comments: related to rDNA projects 497, 500, 501

For your records, make several copies of this document to avoid delays when filing your grant applications with federal agencies.

IBC APPROVAL LETTER

APPENDICIES

1. Gerstenfeld, LC. Editorial: Research Procpective J. Bone and Mienral Research 1999 14:850-855 Osteoponetin in skeletal tissue homestasis: an emerging picture of the autocrine/parcrine functions of the extracellular matrix

2. Carvalho, RS, Schaffer, JL, Bumann, A, and Gerstenfeld LC. Integrin ligands expressed by osteoblasts show preferential regulation in response to both cell adhesion and mechanical stimulation (Unpublished manuscript submitted 1999, Bone)

3. Samuel, E J. Masters Thesis Harvard School of Dental Medicine, Title of thesis "Osteoblast induction of osteopontin expression: repponse to changes in duration and frequency of mechanical strain, and to variation in flow induced shear stress"

Editorial

Osteopontin in Skeletal Tissue Homeostasis: An Emerging Picture of the Autocrine/Paracrine Functions of the Extracellular Matrix

LOUIS C. GERSTENFELD

THE BONE REMODELING CYCLE is intimately involved in the metabolic homeostasis of mineral balance.[1] Bone formation and the remodeling cycle are essential in maintaining the structural integrity of skeletal tissue in response to the mechanical loading to which it is subjected.[2,3] It is likely that remodeling also provides the means of repairing bone tissue damaged as a result of mechanical fatigue.[4] Thus, the skeletal cells (osteoblasts and osteoclasts) that mediate the remodeling process almost certainly are regulated by their mechanical environment. In the article by Terai et al., "Role of osteopontin in bone remodeling caused by mechanical stress"[5] published in this issue of *JBMR*, data are presented that begin to elucidate the molecular events that regulate the remodeling cycle in response to mechanical stimulation. The authors show that induction of osteopontin (OPN) expression by osteoblasts in response to mechanical stimulation provides one of the mechanisms by which osteoclast function is coupled to a response that is regulated by the mechanical loading of the bone tissue. A brief review is presented in this perspective on the state of knowledge concerning the function of OPN in bone and other tissues. This perspective also presents the emerging picture that OPN in particular, as well as other molecules of the extracellular matrix (ECM), play essential roles in skeletal tissue structure and also mediate crucial autocrine and paracrine functions in the regulation of tissue formation and remodeling.

OPN in bone

OPN was initially characterized as one of the predominant noncollagenous proteins that accumulate in the ECM of bone tissue.[6–10] Both its levels of expression relative to other noncollagenous proteins,[11,12] and its quantities of accumulation[6–10,13,14] in the bone demonstrate that it's one of the most prevalent noncollagenous proteins synthesized by osteoblasts. The protein is ~300 amino acids in length and shows a variable size on SDS-PAGE (~70–45 kDa) due primarily to its differing degrees of posttranslational modifications. Sequence comparisons among various species of OPN[15] have identified the conserved protein domains within the mature molecule. These domains include a number of N- and O-linked glycosylation sites, a poly-aspartic acid sequence of 7–10 consecutive aspartic residues, an RGD integrin recognition site, and a number of casein kinase II phosphorylation sites.[16,17]

The OPN gene is ubiquitously expressed in all skeletal tissues during embryogenesis. However, it is not exclusively restricted to skeletal tissues since it is expressed at moderately high levels within developing kidneys.[18] During the in vitro growth of primary osteoblast cultures, OPN expression shows a very early transient peak of expression, after these cells initially attach to their substrate, followed by a continued rise in expression that precedes that of osteocalcin and the mineralization of the ECM.[19–22] Maximal levels of OPN expression are observed when osteoblasts reach full differentiation, and regulatory elements appear to control the promoter activity in both a skeletal tissue-specific manner and in response to AP-1–related activation.[15] During skeletal tissue development in vivo, OPN expression reaches maximal levels of expression during postembryological bone development.[23,24] There is now considerable evidence that osteoclasts also express[25] and synthesize OPN during bone remodeling,[26] suggesting that this protein plays a more important role in postnatal skeletal tissue homeostasis than in initial skeletal tissue development.

Extensive ultrastructural studies of OPN distribution in

Musculoskeletal Research Laboratory, Department of Orthopedic Surgery, Boston University Medical Center, Boston, Massachusetts, U.S.A.

skeletal tissue ECMs illustrate that the protein is distributed throughout the mineralized areas of the bone and hypertrophic cartilage ECM. It is primarily associated with electron-dense areas of matrix-containing aggregates of other noncollagenous proteins that appear between collagen fibrils in the interfibrillar spaces of the ECM.[27-29] One of the most unique aspects of OPN structural organization is its very specific association with boundary surfaces of skeletal tissues. These include the cement lines that demarcate matrix–matrix interfaces, such as that surrounding osteocytes and their canalicular processes and between lining cells and the underlying bone matrix.[30] Other ultrastructural studies indicate that OPN localizes adjacent to the clear zones of resorptive osteoclasts.[31] It has been proposed from these structural and immunocytochemical studies that OPN may have a functional role in providing tissue cohesion within the mineralized portions of the ECM where it is ubiquitously distributed and, likewise, might promote tissue and cellular adhesion at boundary surfaces such as at cement lines, the lamatan limitantes, and sites of matrix resorption.[32]

Numerous functional studies have focused on defining the role of OPN in bone metabolism. It was theorized that, due to its highly anionic and phosphorylated nature and its ability to tightly bind Ca^{2+} and hydroxyapatite,[33,34] phosphorylated proteins in general, and osteopontin in particular, played a role in the initiation and/or regulation of the mineralization process.[33-37] A number of in vitro studies, however, indicate that OPN acts primarily as an inhibitor of mineral nucleation and growth,[38,39] raising the doubt that this molecule plays an important role in the initial deposition of the mineral in the matrix. The identification of the RGD sequence in the OPN molecule and its unique ultrastructural localization at resorption and boundary surfaces[30] within the ECM of mineralized tissues led to other studies focusing on the function of the protein in mediating cell–matrix interactions through its RGD binding with specific integrin receptors.[31,40] Initial in vitro studies demonstrated that OPN promoted cellular adherence of a wide variety of cells including osteoblasts,[13,14] fibroblasts,[41-43] macrophages, T-cells,[44] and osteoclasts.[45] The initial data, indicating that OPN specifically interacted with the $\alpha_v\beta_3$ integrin isotype[40] and identification of $\alpha_v\beta_3$ integrin as one of the predominant cell adhesion receptors on the surface of osteoclasts,[45-47] subsequently led to studies that have documented that osteopontin interaction with this receptor potentiates resorption,[48,49] while antagonism of this receptor interaction with OPN specifically inhibits bone resorption.[50,51] Finally, other data also suggest that both OPN, as well as other RGD-containing extracellular matrix proteins such as fibronectin and bone sialoprotein, may play important roles in directing cellular migration during skeletal growth or in defining the spatial boundaries of mineral deposition within the extracellular matrix.[13,52] Thus, the preponderance of functional and structural data to date suggest that the function of OPN in skeletal tissue is more associated with tissue remodeling and providing cells with positional information within the ECM and less of an inductive role in the mineralization process.

OPN and its role in inflammation and tissue repair

Independent research efforts in both cancer biology and immunology identified that OPN plays an important role in aspects of metastasis and immunological response. OPN is an early, immediate-response gene during both T-cell[53,54] and macrophage activation.[44] In addition, it is an AP-1-inducible gene following phorbol ester tumor promoter treatment in both epithelial[55] and skeletal cells.[56] Finally, it is identified independently as both a serum marker of malignant cell growth[57] and as a component of metastatic tumors from a wide variety of primary tissue origins in mice and humans.[58,59] Numerous studies have shown OPN expression at varying levels in many tissues in conjunction with inflammatory processes induced either in response to infection or tissue injury.[59] Considerable data have been accumulated which show that OPN is associated with a wide variety of pathologies including solid tumors, atherosclerotic plaques,[60] granuloma tissue formation,[61] as well as exogenous pathogen infection.[62] The identification of an OPN interaction with the CD44 receptor also suggests that this molecule may use an alternate cell surface receptor to discriminate varying functions in these differing immunological processes in which it is involved.[63] Common functions that have been ascribed to OPN in these differing pathologies have been its chemotaxis activities and its role in promoting adhesion of monocytes, macrophages, T-cells, and B-cells.[59,62] It is interesting to note the many common aspects of the remodeling cycle of bone in comparison with the biological events associated with inflammation and tissue repair in which OPN may be involved.

Expression of OPN in response to mechanical stimulation and the emergence of the ECM as playing critical autocrine/paracrine functions in matrix homeostasis

Previous studies from several research groups have now shown that *opn* expression is responsive to mechanical stimulation within osteoblasts both in vitro[64-67] and in vivo.[5,68] The current study by Terai et al.[5] provides yet another study demonstrating that OPN expression is induced by physiologically relevant mechanical strains in vivo. OPN induction by mechanical stimulation also occurs in vascular smooth muscle cells[69] and in the egg laying organ of chickens (M. Pines, personal communication). The induction of this gene therefore appears to be a common response to mechanical stimulation in many tissues, and its induction may serve a common function in tissue ECM remodeling that is needed in conjunction with the structural demands of the mechanical environment.

The intracellular mechanisms that transduce mechanical stimuli are still poorly understood. However, a number of different signal transduction mechanisms including Ca^{2+} transients,[70] cAMP/PKA stimulation,[71] alterations in the cytoskeleton,[67,72,73] and integrin mediated[74,75] second signal cascades are all activated by mechanical stimulation of osteoblasts. Examination of the mechanisms of mechano-signal transduction that induced *opn* expression demonstrated that the signal transduction process was a

primary response through the activation of pre-existing transcription factors, dependent on the activation of a tyrosine kinase(s) and protein kinase A (PKA) or a PKA-like kinase, and dependent on microfilament integrity. These studies also demonstrated that mechanical stimuli activated focal adhesion kinase pp125FAK,[67] which specifically transduces signals from integrin receptors. Subsequent studies demonstrated that the cell adhesion to fibronectin but not tissue culture plastic alone mimics the mechanotransduction of *opn* mRNA expression. Furthermore, integrin receptor(s) are involved in mediating the signal transduction processes of both cell attachment and mechanical stimulation, since incubation of osteoblasts with the integrin antagonists such as RGDS partially blocked the induction of *opn* expression in response to both stimuli.[75]

The induction of the *opn* expression, which itself is an integrin ligand, through both integrin occupancy and/or activation by mechanical stimulation, leads to the possibility that the molecules containing integrin binding domains such as RGDS may themselves behave like cytokines. The studies showing that osteoclast recruitment is blocked by the apparent antagonism of OPN function in mechanically stimulated resorption during orthodontic tooth movement[5] provides further evidence supporting the idea that this molecule behaves in many ways like a cytokine. It is interesting to note that fibronectin and bone sialoprotein are also uniquely and separately regulated by mechanical stimulation, while osteocalcin and collagen are not (Carvalho, Schaffer, and Gerstenfeld, unpublished data). Such data provide further evidence that other integrin ligands may have an autocrine or paracrine function in regulating cellular function in the ECM. Indeed, these data show that the composition of the ECM, as regulated by mechanical stimuli, may itself have a specific feedback effect on the osteoblast, thereby providing a self-regulating mechanism of controlling ECM composition.

Although the structural roles of the ECM are easily appreciated, their role in regulatory functions is only now becoming fully realized with the identification of the complexity of cellular receptors that interact with the ECM.[76–79] The cellular logic of using these molecules to carry out regulatory function is both implicit and explicit. The implicit informational content of these molecules resides in the context of their spatial and temporal assembly in the matrix. These molecules therefore provide "positional information." As an example, OPN is synthesized both during early assembly and remodeling steps and later during the period when osteoblasts are synthesizing and mineralizing their ECM after the resorption process has reversed. Thus, during the initial periods of remodeling, transient OPN production provides important regulatory information for the recruitment and migration of osteoclasts into an active area of resorption. The incorporated OPN in the matrix, which had been previously laid down at the end of the last remodeling cycle, subsequently provides the latent structural mechanisms by which osteoclast attachment to mineralized matrix and its activation may be facilitated during a new round of remodeling. Other positional information imparted by OPN includes its role in regulating osteoclast polarity. Explicit information con-

tained in ECM molecules is related to their receptor specificity. The specific ECM protein interactions with unique subsets of cellular receptors provides the means by which specific second signal kinase(s) cascades are activated and cellular responses initiated. As an example, recent data indicate that cellular adhesion to $\alpha_v\beta_3$ ligands activates a specific member of the focal adhesion kinase family, PYK2, and this kinase specifically interacts with *src*.[80] Downstream from the activation of specific signal transduction cascades are wide-ranging numbers of cellular response, including specific gene activation, stimulation of cellular replication, or induction of apoptosis.[77]

While this review has focused only on the function of one ECM protein, OPN, a brief description is provided of other data that demonstrate the central role of the ECM in providing regulatory signals to cells. It has been recognized for some time that collagenous matrix itself provides crucial signals during the temporal differentiation of osteoblasts and experimentally this may be observed since osteoblasts growing in the absence of ascorbate (an essential cofactor in the synthesis of collagen) fail both to develop a collagenous matrix and to progress in their differentiation.[20,81,82] Recently, a number of studies have shown that integrin ligation of $\alpha2$ receptors is necessary for the ascorbate acid mediated induction of the osteogenic phenotype in cultures MC3T3 E-1.[83–85] More specifically, these studies have identified both focal adhesion kinase (FAK) and mitogen-activated protein kinases as mediating the ascorbic acid induction. In one study, the specific activation of pre-existent CBFA transcriptional factor has been shown to be the mechanism of action by which the matrix induces osteogenic progression.[85] In conclusion, the data presented in this perspective present the convergence of several different lines of research that provide an emerging picture that the ECM contributes its own set regulatory signals crucial for mediation, cellular formation, remodeling, and repair.

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THE PREDOMINANT INTEGRIN LIGANDS EXPRESSED BY OSTEOBLASTS SHOW PREFENTIAL REGULATION IN RESPONSE TO BOTH CELL ADHESION AND MECHANICAL STIMULATION

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Summary: Previous studies from this laboratory demonstrated that both mechanical stimulation and cell adhesion induced the expression of osteopontin *(opn)*, a predominant integrin ligand expressed by osteoblasts (Carvalho et al., 1998). The present study examined if osteoblast expression of other integrin binding proteins such as fibronectin *(fn)* and bone sialoprotein *(bsp)* would be similarly responsive to mechanical stimulation and cell adhesion. All three genes showed 3 to 4 fold maximal induction in response to a single two hour period of an applied dynamic spatially uniform biaxial strain of 1.3% strain at 0.25 Hz. Each gene however had a different time course of induction with *bsp fn* and *opn* showing their maximal response at one three and nine hours respectively after the stimulation period. In contrast the levels of both collagen type I and osteocalcin mRNAs were unaltered over the same time course of examination. The effect of cell adhesion on the expression of these genes was seperately examined to determine if this stimuli would mimic the effects of the mechanical stimulation. All three genes also showed comparable levels of induction in response to adhesion on the fibronectin coated surfaces in comparison to non-coated surfaces with maximal levles of induction seen for *bsp* and *opn* at 24 hours after plating while *fn* showed maximal levels of stimulation at 8 hours. Interestingly while both *onp* and *fn* mRNA expression returned to base line after cell adhesion on fibronectin *bsp* mRNA levels remained elevated. Examination of the signal transduction pathways that mediated the gene expression in response to cell adhesion on fibronectin coated surfaces showed that both genistein and cycloheximide inhibitied the induction of all three genes demonstrating that a tyrosine kinase was involved in the mechanical stimulated induction of these genes and new protein synthesis was a prerequisite to this process. In contrast the PKA specific inhibitor H-89 only ablated the induction of *fn* expression. Depolymerization of either microtubules or microfilaments with

colchicine or cytochalasin D respectively had little effect on the over all expression of these gene in response to cell adhesion indicating that the adhesion mediated phenomenon was not dependent on cytoskeletal integrity. In summary these results show that both mechanical stimulation and cell adhesion specifically stimulated the expression of integrin binding proteins. These results further demonstrate that while there are common features in the signal transduction processes that mediated the induction of these genes, each gene was separately induced by unique mechanisms.

INTRODUCTION

The mediation of cellular responses to mechanical stimuli depends in part on the recognition and interaction of selected cell-surface receptors with the extracellular matrix. Integrins are one class of cell surface receptors that mediates cell adhesion to the extracellular matrix. The integrins are a family of are heterodimeric membrane receptors composed made up of multiple α and β isotypes. Variations in the extracellular domains of the individual isotypes of integrin receptors imparts their specificity for specific extracellular matrix proteins (Hynes, 1992; Miyauchi, 1995). The interaction of many integrin isotypes with their specific ligands is mediated by their recognition of the amino acid sequence arginine-glycine-aspartic acid-serine (RGDS) (Hynes, 1992). It has been demonstrated that the binding of specific adhesion proteins to their integrin receptors generates a cascade of intracellular signals that are responsible for the regulation of a wide variety of cellular responses (Damsky and Werb, 1992). Such interactions facilitate the appropriate functioning of essential cell processes such as cell adhesion, cell migration, and facilitates the survival of many cell types. These many functions in turn are mediated through integrin receptor activation of specific signal transduction mechanisms or by their facilitation of structural alterations in the cellular cytoskeletal architecture(Juliano and Haskill, 1993).

In bone tissue, osteoblasts express high levels of several different RGD-containing proteins, the most predominant being osteopontin, bone sialoprotein and fibronectin (Gotoh et al. 1995, Puleo and Bizios, 1992). Osteopontin has been shown to interact with both osteoblasts and osteoclasts (Oldberg et al., 1988; Gotoh et al., 1990; Ross et al., 1993), and is thought to play a role in mediating osteoclast resorption of bone tissue (Reinholt et al., 1990; Denhart and Guo, 1993). The expression of osteopontin is seen concurrently with alkaline phosphatase, and it has

been identified as an early marker of osteoblast differentiation (Gerstenfeld et al., 1990) Bone sialoprotein is another specific integrin ligand that is expressed by osteoblasts having a very restricted expression to only cells within the skeletal lineage and is seen predominantly in areas of mineralized growth cartilage and osteoid (Chen et al., 1994; Yang and Gerstenfeld, 1996; 1997). Bone sialoportein has been shown to initiate calcification through its binding properties to collagen, calcium and hydroxyapatite (Hunter and Goldberg, 1994). Unlike the former two proteins, fibronectin is expressed ubiquitously in most connective tissues, however this protein appears to play an important role in the mechanisms of cell attachment, spreading and migration during early osteoblast differentiation (Winnard et al. 1994). In addition to the RGDS peptide fibronectin contains a synergistic adhesion site to the RGD sequence and the two sites in the type III connecting segment of the whole molecule: the CS1 portion and the arginine-glutamic acid-aspartic acid-valine (REDV) sequence within the CS5 portion of fibronectin (Puleo and Bizios, 1992). Some studies suggest that fibronectin also acts as an activator of cell adhesion rather than a direct adhesion molecule (Curtis et al., 1992) thus fibronectin may mediate multiple interactions and response by cells. Currently, there is enough evidence to suggest that the RGD-containing proteins osteopontin, bone sialoprotein and fibronectin are integral parts of cellular differentiation and migration events during skeletal growth and/or in the initiation of spatial deposition of mineral in the extracellular matrix (Curtis et al., 1992; Hunter and Goldberg, 1993; Gerstenfeld et al., 1995; Schaffer et al., 1996).

Previous studies have shown that both the mechanical environment of osteoblasts and cell adhesion induces osteopontin gene expression (Toma et al., 1997, Carvalho et al., 1998). These studies then suggest that interactions of cells with the extracellular matrix are integral components to the mediation of these stimuli however stimulation through receptors may occur both through

both occupancy of receptor as well as deformation through the engagement of the recpetors with matrix attachment (Miyauchi et al., 1995). Recognized events of specific reverse phosphorylation of certain proteins appear to regulate various intracellular pathways exclusively upon cellular attachment (Guan et al., 1991). However, aggregation of receptors alone may not be sufficient to induce genomic effects. This raises the possibility that signal transduction may be dependent on the matrix composition and that the cellular matrix components themselves may function as autocrine factors.

Mechanical or receptor-ligation stimulations appear to be dependent on the positional parameters provided by the matrix. Application of mechanical strain in cells of osteoblastic lineage showed that only those cells attached to specific ligands such as fibronectin, increased their DNA synthesis (Wilson et al., 1995). Such modulations may involve cytoskeletal integrity and its relation with the integrin receptors. It has been hypothesized that cellular shape changes determine signal trasnduction pathways through the direct deformation of cellular membranes and reorientation of the microfilament network affecting integrin behavior (Ingber, 1991). However, Meazzini et al. (1997) have suggested that physical alteration of ECM proteins may change integrin conformation, facilitating the activation of specific signal transduction molecules which in turn regulate cytoskeletal arrangement and cellular response. This contrasting issue may be analogous to the role of the extracellular matrix proteins themselves as initiators of cellular activation. In order to investigate this parameter in skeletal tissue, it is important to understand the behavior of selected extracellular matrix proteins in response to processes of cellular adhesion and mechanical stimuli. Therefore, in this study we have examined the expression of mRNA levels of *opn, bsp* and *fn* following both mechanical stimulation and cell-ligand binding. The signal transduction pathways for these proteins following cellular attachment were also studied.

MATERIALS AND METHODS

Materials

All tissue culture supplies, cytochalasin D, colchicine, cycloheximide were from Sigma Chemical Company, St.Louis, MO. H89 genistein was from LC Laboratories, Woburn, MA. Nylon membranes for Northern blots were from Biotrans, ICN Corp. Aurora, OH

Cell Culture

Seventeen-day embryonic chicken calvaria osteoblasts were and grown in culture as previously described (Gerstenfeld et al., 1988). These cells were plated at a density of 2×10^6 cells in 100 mm tissue culture dishes coated either with purified FN (1mg/ml) as previously described (Schaffer et al., 1994) or uncoated plates. Cultures were grown for two weeks until they reached confluence in minimum essential media supplemented with 10% fetal bovine serum (FBS). The medium was changed to BGJb supplemented with 10% FBS with the addition of 10mM β-glycerophosphate and 12.5 µg/ml ascorbic acid. All analyses were performed on at least three separate preparations of cells and all data is presented as a percent increase in expression over that of the controls which were determined from parallel cultures grown under identical conditions. All error bars represent the S.D. of the determinations from separate experiments and the number of replicates that were used for each measurement is denoted in each figure.

Mechanical Stimulation / Attachment Assays

The mechanical stretch apparatus used for these experiments was previously described (Schaffer et al., 1994). The design of the device imposes a verified temporal and spatial displacement profile to an optically transparent elastomeric membrane in which the strain magnitude was experimentally demonstrated to be homogeneous and isotropic (i.e. radial strain = circumferential strain = constant over the culture surface). A polyurethane membrane (a generous

gift of Dow Chemical Corporation, Midland, MI) was used in the culture dishes allowing for a constant 1.3% uniform biaxial strain at 0.25 Hz to be applied for a single two hour period. For each experiment non-stimulated controls were carried on cultures at the same time and from the same preparation of cells grown at identical conditions as the mechanically stimulated cultures. In all experiments for mechanical stretch, determinations were carried out six hours after the end of the two hour period of active cellular stimulation. For the attachment/integrin ligation assays the cells were allowed to attach to FN coated (1mg/ml) dishes (used with the same concentration as those of mechanical stimulated cultures) for 24 hours. FN served as the basic ligand and uncoated plastic plates served as controls.

Signal Transduction Studies

Signal transduction pathways that mediate the cell responses of mechanical stimulation and/or attachment/ligation were investigated by the use of specific chemical inhibitors. The final concentration for each of these compounds was: 50 mM cycloheximide, 20 mg/ml genistein, 1 μM H89 (Sigma), 50 μM cytochalasin D (Sigma) and 1 μM colchicine (Sigma). Cycloheximide and genistein were incubated for 30 minutes, while cytochalasin D was incubated for 1 hour and colchicine for 6 hours. Controls were separately determined for each compound, in cultures treated identically with the various compounds but in which the cells were either not mechanically stimulated or were attached to uncoated dishes.

Isolation and Analysis of RNA

Total RNA was isolated using tri-ReagentTM (Molecular Center, Cincinati, OH) according to the manufactures instructions. RNA was resolved on 1% agarose gels containing 2.2 M formaldehyde (Toma et al., 1997) and 5 mg of total RNA was loaded per gel/lane. Chicken cDNA's used for these studies were pro α1[I] collagen, (Lehrach et al

1979)osteocalcin,(Nuegelbaurer et al .1995) osteopontin, (Moore et al., 1990) and bone

sialoprotein. (Yang et al. 1995) Northern blots with P^{32} cDNA-labelled probes were carried out at

65°C in 2.5 X SSC, 50 mM Na phosphate buffer, made 100 µg/ml single stranded salmon sperm

DNA, and for 18 to 24 hours in a rotating hybridization oven (Robins Scientific, Sunnyvalle, CA).

Autoradiograms were quantified using an LKB Ultra II scanning densitometer (LKB, Broma,

Sweden) and values were normalized 18 S ribosomal RNA obtained by hybridization of each blot

to a conserved nucleotide sequence probe of 18 S ribosomal subunit (Ambion Corp., Austin, TX).

All analysis were performed at least three times and all data is presented as a percentage in

expression over that of the control which were determined from parallel cultures. All data were

evaluated as a mean ± 2 standard deviations with a minimum of three experiments from different

populations of primary cells and appropriate statistical analysis were performed.

RESULTS

Osteoblast-adhesion and mechanical stimulation increase levels of RGD-containing

proteins: Initial studies were carried out to assess the expression of mRNA levels for

osteopontin, bone sialoprotein and fibronectin following osteoblast either cell adhesion or

mechanical stimulation. The induction of mRNA expression was measured for three predominant

integrin binding ECM proteins expressed by osteoblasts: fibronectin, bone sialoprotein and

osteopontin. Fibronectin was used as the main adhesion substrate because of its anchoring

property for cells in the extracellular matrix (Pierschbacher et al., 1981). The temporal profiles of

opn expression were shown to peak at 24 hours. This induction was 3 to 4 fold from above that

of control samples. At later time periods beyond 24 hours, there was a sharp reduction in *opn*

expression, which returned to its baseline levels of expression. (Figure 1) Bone sialoprotin

expression followed a similar profile to that seen for *opn* also peaking at 24 hours (3 fold),

however, there was no marked decrease in the expression of *bsp*, which remained elevated (2 fold) at even at 48 and 72 hours (Figure 2). In contrast, the expression of *fn* mRNA started at significantly higher levels when compared to the other two mRNAs (Figure 3). Fibronectin expression peaked relatively early at 8 hours from the onset of the stimulation (2 fold), and showed s sharp reduction to its baseline levels soon thereafter.

The expression of these mRNAs was then examined after the application of mechanical stimulation. As expected, mechanical stimuli of osteoblasts increased *opn* expression by 2 to 3 folds up to a maximum at 9 hours post stretch (Figure 1). This clearly contrasted with adhesion mediated induction of this gene which showed a peak in *opn* expression at 24 hours. In case of *fn*, mechanical stimuli also showed an increase in expression peaking at 3 and 6 hours for the onset of the stimuli (Figure 3). It is interesting to note, however, that mechanical stimuli was inhibitory for *bsp* expression. In Figure 2 *bsp* levels started at 2 fold of control samples at 1 hour post stretch and quickly decreased to baseline levels at 9 hours post stretch.

These results would suggest that integrin binding ECM molecules are selectively responsive to stimulation either via mechanical stimulation or cell adhesion mediated signal transduction. Two other prevalent ECM genes collagen type I and osteocalcin were then examined as a comparison to these RGD integrin ligands. Both collagen type I (*colI*) and osteoclacin (*oc*) mRNAs were then examined after mechanical stimulation. Interestingly neither one of these genes showed alterations in there expression when the cells were subjected to mechanical stimulation (Figure 4).

Different matrix proteins require different signal transduction pathways following cell adhesion: In order to further understand if the signal transduction processes that mediated the induction of the three RGD genes were the same pharmacological inhibitors of specific signal

transduction pathways were used. It appeared that from the slow increase in *opn* levels following

cell adhesion that this protein would be a secondary event to down stream to other genomic

changes. Indeed, the compound cycloheximide, a known inhibitor of *de novo* protein synthesis,

blocked the induction of *opn* mRNA expression following adhesion to fibronectin (Figure 5A).

Cycloheximide was also used to inhibit the expression levels of *fn* and *bsp* genes. Figure 5B and

5C show that these genes were also dependent on new protein synthesis following cellular

adhesion. Even though the maximum levels of *fn* occurred at 9 hours post cellular adhesion, this

finding was consistent with the relative long period for maximum induction of *opn* and *bsp*

(Figures 1- 3).

Previous observations had shown that changes in *opn* mRNA expression in response to

mechanical stimulation were dependent on the integrity of the microfilament structure of the cell

(Toma et al., 1997). The role of the cytoskeleton in the signal transduction pathways for each of

these mRNAs was examined in these studies. Incubation of the osteoblast cultures with the

microfilament depolymerizing agent cytochalsin-D, did not affect the expression of any of the

mRNAs (Figure 5). Similarly, cultures treated with colchicine a microtubule disruption agent was

also not capable of inhibiting the levels of these mRNAs below those of control levels (Figure 5).

Finally, specific inhibitors for second messenger systems were used. The use of genistein,

a potent inhibitor of tyrosine kinase phosphorylation was shown to significantly inhibit the

expression of *opn*, *bsp* and *fn* mRNAs. This finding has also been observed previously for opn

expression in mechanically stimulated cells (Toma et al., 1997; Carvalho et al., 1997). The

pharmacological inhibitor of PKA-like kinases H-89, also caused an inhibiton of *fn* levels, but not

effect the expression of *opn* or *bsp*. These findings suggest that the stimulation in expression of

the RGD-containing extracellular proteins studied here in response to cell adhesion is uniquely dependent on the activation of specific subsets of kinases.

DISCUSSION

The present study provides evidence that the mechanisms of cellular adhesion and mechanical stimulation are perceived by the osteoblast in a selective fashion through different mechanisms of transduction depending on the extracellular matrix ligand. These results further confirm that cellular adhesion and mechanical stimulation are separate events. The stimuli applied to the cells are not constant. Even though, many studies assume that the nature of stimuli such as mechanical stretch is of an uninterrupted fashion, the cell may receive many forms of inputs simultaneously (Ingber, 1997) with a hierarchical organization and information recording. These inputs are capable of regulation intracellular functions, and many signaling mechanisms reported to change by mechanical stimulatory influences do also change by hormone binding, cellular adhesion, etc... Until very recently, all the efforts to understand mechano-transduction centered on the study of mechanical signals and its associated pathways of response. Subsequently, little attention was placed on other forms of stimulations that may influence the nature of the response following either cell stretching or changes in fluid flow conditions. This appears to be the case for cellular adhesion as a pre-event to any physical stimulation of the cells. We have reported that even though cellular stretching caused a specific set of regulatory intracellular signaling, these were distinct from those of cellular adhesion alone in the same cell types (Carvalho et al., 1998). The challenge has been to explain how these signals are integrated and how the cellular responses from each stimulus contribute to the responses of the next stimuli.

In bone matrix, RGD-containing glycoproteins including osteopontin, bone sialoprotein and fibronectin are presumed to interact with adhesion receptors in the surface of bone (Grzesik

and Robey, 1994). Studies suggest that that these ECM components may directly affect gene expression (Pienta et al., 1991), which also takes place following mechanical stimulation (Resnick et al., 1993; Toma et al., 1997, Carvalho et al., 1998). When considering adhesion separately from mechanical stimulation, one needs to take into account the effects of former over the latter. The speculation at this point may indicate that adhesion or integrin-ligation act as a "primer" prior to any stretch response, or as a cellular adaptation to the environment with its own sets of signals. In particular, it is interesting to speculate that the effects of stretching cells that are in the process of adhesion enhances the formation of new receptor-ligand bonds. According to Lauffenburger and Linderman (1993), mechanical stimulation can alter the kinetic regulation of this reaction, thus interfering with binding rate. Similarly, in this laboratory we have demonstrated that specific tri-peptides RGD inhibited adhesion (receptor ligation) and stretch responses (Carvalho et al. 1998). Wilson et al. (1995) presented evidence that RGD peptides, fibronectin and certain integrin antibodies disrupted integrin-ligand interaction, which in turn ablated strain mechano-transduction responses, without disrupting adhesion of the same cells. It has been proposed that receptors such as integrins can behave as a homeostatic system for modulating the ECM structure and organization in response to the structural needs of the cell (Werb et al., 1989), contrary to previous observations shown in chondrocytes referring to the binding of RGD peptides inhibiting the attachment to FN, OPN and BSP (Loeser, 1994). Moreover, RGD peptides appear to inhibit the adhesion of osteoblasts and osteocytes to the RGD-containing proteins FN, vitronectin, fibrinogen, laminin and OPN (Puleo and Bizios, 1992; Aarden et al., 1996). It was unclear if the same tri-peptides like RGD and conceivably other blockers would disrupt the mechanical stimulation without disrupting adhesion. However, recent studies suggest that domains other than RGD ones on OPN and BSP may promote cellular attachment (McNeil et al., 1995). Previous

investigations suggested that bone may utilize multiple receptor systems (both RGD-dependent and independent) in interaction with cellular ligands.

While stimulations should be transferred across points of contacts where the cells are anchored to the extracellular matrix (ECM), due to the anchoring nature of the ECM, any changes in physical properties such as stiffness will change cellular response. This is due to the dissipation of stress and regulation of soluble events. By changing the constitution of the ECM, one would expect to influence a particular type of response. The importance of extracellular interactions for the process of mechano-sensation has been discussed by Du et al. (1996) in recent experiments demonstrating that isolated genes of the touch receptor neurons in C. elegans encode extracellular proteins. Du et al. (1996) have also hypothesized that the ECM mediate the anchoring properties of specialized cells enabling their mechano-sensory response. For now, ECM proteins may themselves be regulated by selectively different pathways of response. Curtis et al. (1992) showed that FN can activate and indirect mechanism of adhesion that does not involve fibronectin itself. Thus, ECM protein expression by integrin-FN binding can contribute to FN regulation supporting the idea that ECM proteins may function as structural autocrine factors.

Davies et al. (1993) has suggested that the transduction of forces in anchorage-dependent cells is due to a combination of force transmission via the cytoskeleton and transduction of the physical forces to biochemical signals at mechano-transducers sites. This concept was also suggested by Ingber (1997) stating that changes in the cells following stretching are known to occurs through the discrete adaptations of the filamentous network that provide the cytoplasm's principal mechanical strength and the path for signal transduction between the ECM and the nucleus. In order for these adaptations to occur it is necessary that cooperative interactions between micofilaments, intermediate filaments and microtubles exist. However, that does not

depend on the structural integrity of these structural elements; at least not concomitantly. This is shown here following the selective inhibition of microtubules and microfilaments. According to Table I, opn expression was inhibited following cytochalsin-D treatment, but was not affected when colchicine was used. By contrast, inhibition of cytoskeletal components did not change the expression of *bsp* pr *fn* mRNA. Genistein inhibited the expression of all RGD-containing proteins studied, in contrast to results by Ishida et al. (1997) which did not report any inhibition of shear-stress mediated tyrosine phosphorylation. This suggests that a tyrosin kinase(s) are involved in the signal transduction of these proteins and this seems to be a common mechanism in both stretch and adhesion-mediated responses. Finally, PKA inhibition through H-89 treatment demonstrated a selection for the stimulation applied. For *opn* and *bsp*, H-89 inhibited the effects of stretch induction, but not those of adhesion; and for *fn*, H-89 inhibited the effects of adhesion but not those of stretch. In our experiments we did not see any changes in non-RGD containing proteins collagen type I and osteocalcin. These results are contradictory to the results for Yasuda et al. (1996) that found an increase in the levels of $\alpha(I)$ collagen gene following stretch/relaxation experiments in rat glomerular mesanglial cells.

This differentiation between stretch and adhesion appears to follow unique mechanisms. However, we can not speculate on the nature of the integrin receptors generating the responses found here. It has been demonstrated, however, that opn appears to interact with integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$ (Hu et al., 1995) even though, the nature of the ligand-receptor interaction will determine the effect that the ligand will produce in the cell. For instance, while $\alpha_v\beta_3$ deficient cell populations were not capable of migrating to opn, these same cells did migrate significantly in response to fibronectin and vitronectin (Liaw et al., 1995). Once inside the cell, the transduction of the signal depend on mediators ranging from ions and G-proteins to specialized kinases and

phosphatases. The transduction of the mechanosignal at the cellular membrane leads to a cascade of downstream signaling events, many of which mediated by tyrosine kinases, which in turn phosphorylate other kinases (Berk et al., 1995). Kinases that have been associated with the mechanotransduction include the mitogen-activated protein kinase (MAPk) following stretch in cardiac cells (Yamazaki et al., 1993) and fluid flow in endothelial cells (Tseng and Berk, 1993). Berk et al. (1995) suggested that such responses are part of a multiplicity of pathways divided in calcium dependent and calcium independent pathways. (Ishida et al., 1997) The presence of calcium is important for the activation of a putative shear stress receptor (membrane level) regulating a pertussis toxin-sensitive G protein-coupled K^+ channel (SSR)(Ohno et al., 1993) and the enzyme phospholipase C (Nollert et al., 1990). The levels of PIP_2, in turn will be regulatyed by rho, a small GTP-binding protein (Chong et al., 1994). The calcium-independent pathway involves the activation of MAPk (Berk et al., 1995), however, other calcium-independent tyrosine kinases such as src and FAK may also be involved in the shear stress transduction

In this study we have shown that integrin-ligation to RGD-containing proteins has stimulated gene expression, similarly to that of mechanical stretch. It has been shown that the activation of the integrin receptors with ECM ligands causes a recruitment of tyrosine kinases (Schaler and Parsons, 1994). Integrin receptors have been described as a candidate as the mechanical stimulation receptor (Ingber et al., 1994; Ishida et al., 1997). Activation of integrins has been shown to induce phosphorylation of FAK at focal adhesion complexes (Schaller et al., 1994). In addition, other proteins within these focal adhesion contacts will also be phosphorylated, such as paxillin and src when exposed to flow (Girard and Nehem, 1993; Bull et al., 1994). Our laboratory has shown that FAK phosphorylation was regulated by mechanical stretch (Toma et al., 1997). Our work also suggests that the disruption of microtubules does not

affect the expression of any gene studied following mechanl stretch. This is an interesting finding as it relates to MAPk. This kinase has been shown as the earliest signal activated by flow at physiological stress (Tseng and Berk, 1993), and is also known as a microtubule-associated kinase (Sabe et al., 1994), suggesting a role in the cytoskeleton. However, since disruption of microtubules did not affect gene expression following stretch, one may speculate on the role of MAPk as a stretch-dependent kinase. On the other hand, we have observed that adhesion alone in the presence of the microtubule-disrupting drug colchicine, did not stimulate OPN expression in particular. Thus, it is conceivable that MAPk plays a role in this mechanism, since this kinase has been shown to be activated by cell binding to fibronectin (Morino et al., 1995). It has been suggested that activation of integrins is associated with the same signal events that occur when cells are exposed to flow (Vuori and Ruoslahti, 1993; Schwartz and Deninghoff, 1994; Beck et al., 1995). The complexity of such response can not be understood if the responses of stretch and adhesion are not taken into account individually.

Integrin-ligation is thought to stimulate the same signal events as mechanical stretch (Berk et al., 1995). Indeed, we have shown here that this is the case, even-though the mechanisms that mediate both responses are uniquely different. If integrins are the mediators for mechano-transduction in both forms of activation, then there maybe several different integrin receptors acting in concert with other sensors that are specific to the activation in question. The dependency of stretch effects on RGD-containing proteins in this study and the lack of response in either collagen type I or osteocalcin further demonstrate an active role of integrins in adhesion and stretch. It is clear that integrins and focal contacts play important roles in mechano-transduction. It remains, however, to be determined how the mechanisms of adhesion cross talk with those of

mechanical stimulation and which kinases and second signals are common in regulating

downstream events prior to any activation in gene expression.

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FIG. 1. Effect of cell adhesion and mechanical stimulation on the temporal expression of
opn **mRNA expression by osteoblasts. A)** Northern blot analysis of osteopontin mRNA
expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of
induction in response to mechanical stimulation is seen on the right. The expression of the 18S
rRNA is seen in the lower panel of each figure. The graphic representation of the temporal
expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24
hour period of attachment relative to the control samples at identical times. The graphic
representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours
post mechanical stimulation. Controls=(C) and stretched samples =(S). All data is presented as
percent induction of expression of the mRNAs relative to their control samples. Error bars are
the S.D. determined from at least three experiments..

FIG. 2. Effect of cell adhesion and mechanical stimulation on the temporal expression of
bsp **mRNA expression by osteoblasts. A)** Northern blot analysis of bone sialoprotein mRNA
expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of
induction in response to mechanical stimulation is seen on the right. The expression of the 18S
rRNA is seen in the lower panel of each figure. The graphic representation of the temporal
expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24
hour period of attachment relative to the control samples at identical times. The graphic
representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours
post mechanical stimulation. Controls=(C) and stretched samples =(S). All data is presented as
percent induction of expression of the mRNAs relative to their control samples. Error bars are
the S.D. determined from at least three experiments..

FIG. 3 **Effect of cell adhesion and mechanical stimulation on the temporal expression of *fn* mRNA expression by osteoblasts.** A) Northern blot analysis of fibronectin mRNA expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of induction in response to mechanical stimulation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24 hour period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours post mechanical stimulation. Controls=(C) and stretched samples =(S). All data is presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the S.D. determined from at least three experiments..

FIG. 4. **Effect of mechanical stimulation on the temporal expression of *coll* and oc mRNA expression by osteoblasts.** Northern blot analysis of osteocalcin and collagen type I mRNA expression following mechanical stimulation of osteoblasts are denoted in the figure. The graphic representation of the temporal expression for these mRNAs is shown by the times of 1, 3, 6 and 9 hours post stimulation. All the panels show the percent induction of expression of the steady mRNA levels relative to their control samples. Controls=(C) and stretched samples =(S). Error bars are the S.D. determined from at least three experiments

FIG. 5.   Effect of pharmacological inhibitors on the mRNA expression of *opn*, *bsp* and *fn* by osteoblasts in response to cell adhesion. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *bsp fn* and *opn* mRNAs in response to cell adhesion were examined. Analysis of the expression of these mRNAs was undertaken following the adhesion of the osteoblasts. Cells were treated with the microfilament depolimerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89) and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18 S r RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the S.D. of the three experiments.

Table I. Comparison of signal transduction pathways for the mRNA expression of *opn*, *bsp*, *fn* between cell adhesion and mechanical stretching.

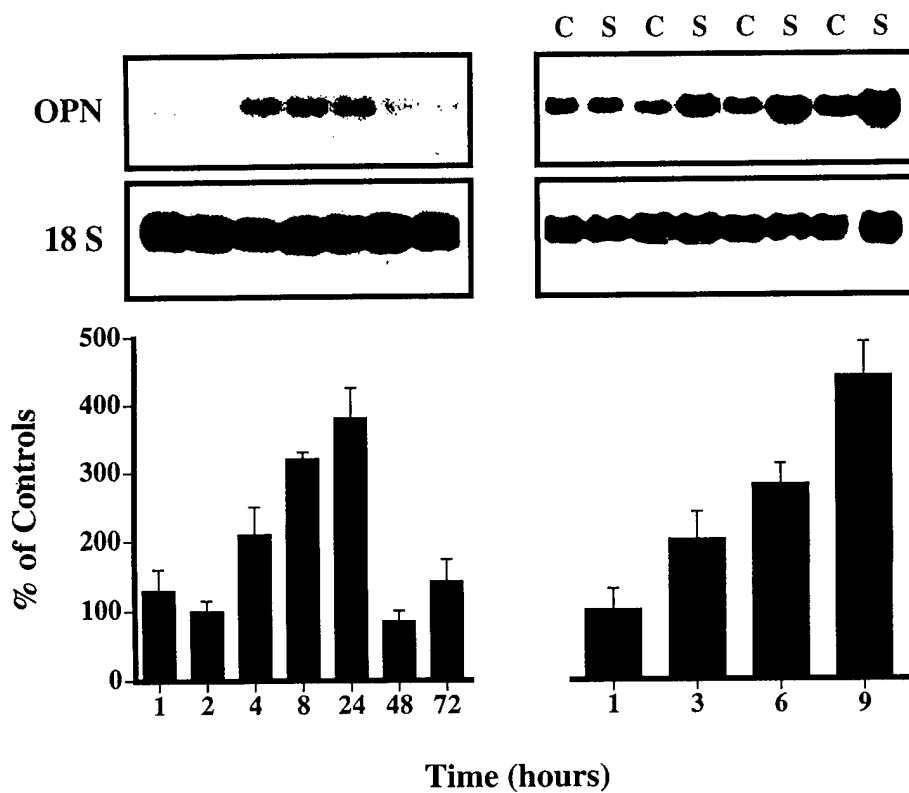| Genes	*Opn*		*bsp*		*fn*	
Stimulation	Adhesion	Stretch	Adhesion	Stretch	Adhesion	Stretch
Time after peak expression	24 hrs	9 hrs	24 hrs	1 hr	8 hrs	3 hrs
de novo protein synthesis	yes	No	yes	no	yes	no
Tyrosine kinase-mediated	yes	Yes	yes	yes	yes	yes
PKA-mediated	no	Yes	no	yes	yes	no
Requires microfilaments	no	yes	no	no	no	no
Requires microtubules	yes	no	no	no	no	no

FIGURE 1

FIGURE 2
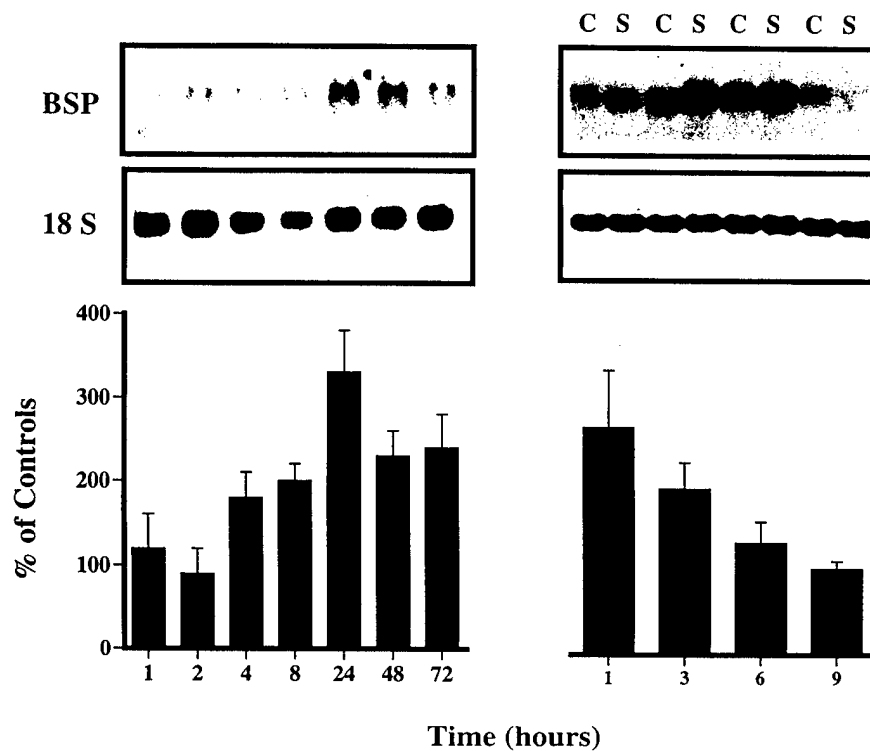
FIGURE 3

FN

18 S

C S C S C S C S

% of Controls

400
300
200
100
0

1 2 4 8 24 48 72

1 3 6 9

Time (hours)

FIGURE 4

C S C S C S C S

C S C S C S C S

OC

Col I

18 S

18 S

% of Controls

200
150
100
50
0

1 3 6 9

1 3 6 9

Time (hours)
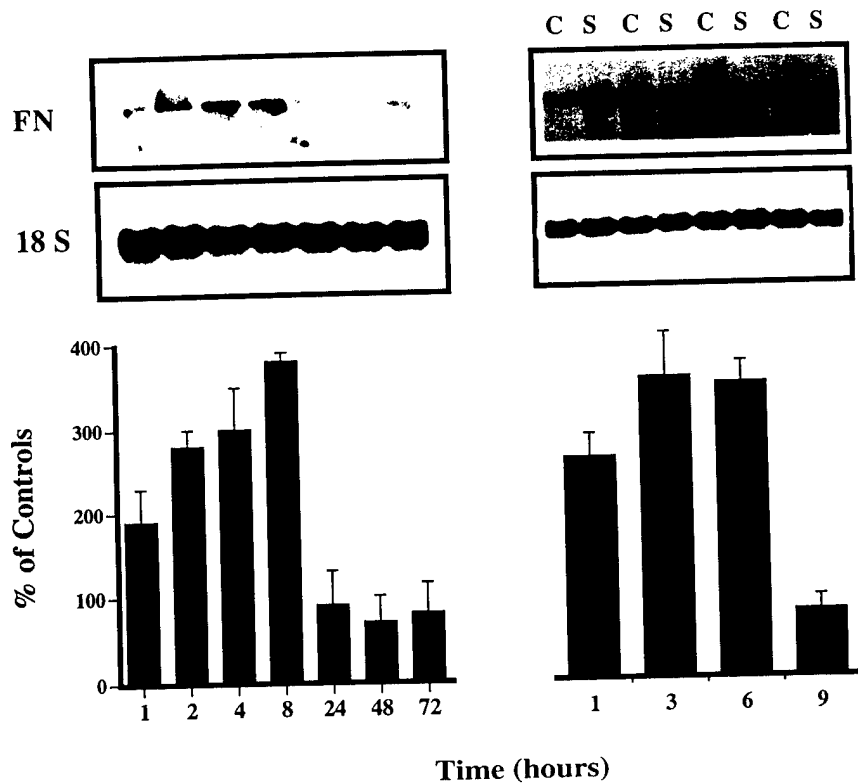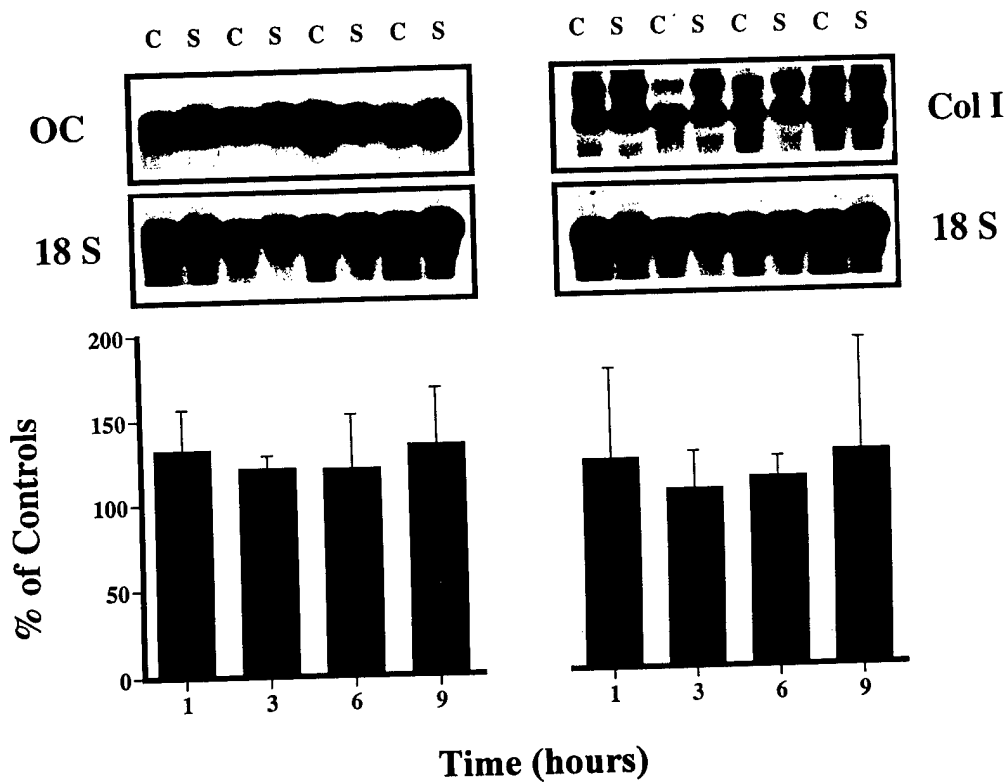
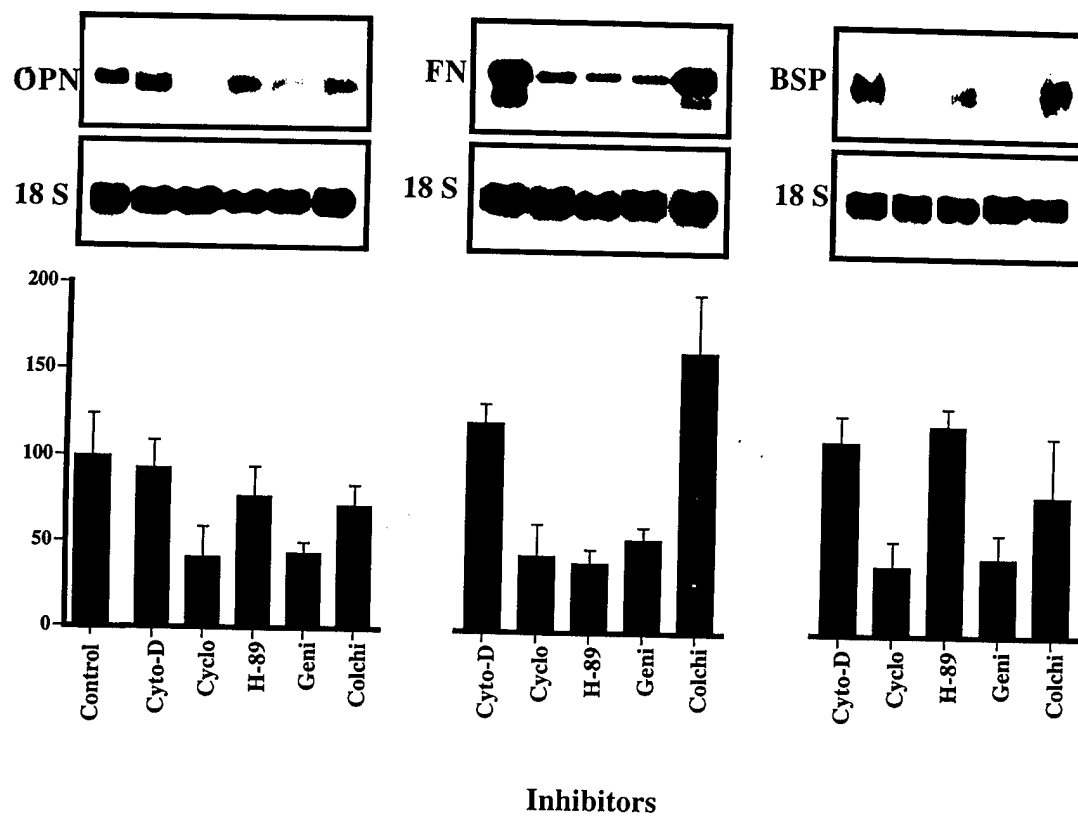FIGURE 5

OSTEOBLAST INDUCTION OF OSTEOPONTIN EXPRESSION: RESPONSE TO CHANGES IN DURATION AND FREQUENCY OF MECHANICAL STRAIN, AND TO VARIATIONS IN FLOW-INDUCED SHEAR STRESS

by

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ABSTRACT

Expression of osteopontin (OPN), an integrin binding protein of bone, is induced by mechanical stimulation of osteoblasts (Toma et al., J. Bone Miner. Res., 12:1-12, 1997). The purpose of this study was to further analyze several parameters that modulate this process, specifically duration and frequency of mechanical perturbation, as well as the effect of flow-induced shear stress on OPN expression. Using a dynamic membrane stretch model, embryonic chick calvarial osteoblasts were subjected to a spatially uniform biaxial strain (1% applied strain) for periods of 1, 5, 15, 30 minutes and 1, 2, 4, 8 hours, and then collected 8 hours after strain initiation. Northern blot analysis of mRNA and quantification via hybridization with a radiolabeled cDNA probe encoding chicken OPN were carried out, and a maximal increase of 145% of control was detected for 8 hours of perturbation ($p < 0.05$). Osteoblasts were then subjected to varying strain frequencies (0.1, 0.25, 0.5, 1 Hz) for 8 hours, and OPN expression was found to increase significantly ($p < 0.05$) in conjunction with increasing frequency. In the third part of the study, the previous range of strain frequencies was used, and the cells were divided into two equal subgroups located peripherally (experiencing greater shear stress) and centrally (experiencing lesser shear stress) on the circular stretch membrane. Analysis of OPN mRNA production revealed a significant difference ($p < 0.05$) between central and peripheral osteoblasts only for the 1 Hz group, with greater OPN expression by centrally located cells. These data indicate that elevations in the duration and frequency of mechanical perturbation induce significant increases in osteopontin expression, while an inverse relationship exists between the level of fluid shear stress and OPN induction at higher frequencies.

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INTRODUCTION

Bone is one of the hardest tissues in the human body, as well as one of the most durable. In its capacity as the principal component of the adult skeleton, bone functions to provide internal support for connective tissue structures, to serve as a lever against muscular contraction, to protect vital organs in the cranial and thoracic cavities, and to house the bone marrow, where blood cells are formed. Bone also serves as a dynamic mineral reservoir for ions such as calcium and phosphate, which are crucial for the proper functioning of numerous tissues (Kaplan, 1987).

Bone is a specialized connective tissue consisting of an intercellular calcified matrix, as well as three different cell types which contribute in different ways toward maintaining its structural integrity and efficiency. Osteoblasts are bone surface cells responsible for synthesizing the organic components of bone matrix (*i.e.* type I collagen, proteoglycans, glycoproteins). During active matrix synthesis, these cells have a cuboidal to columnar morphology, a basophilic cytoplasm, and a high alkaline phosphatase activity. Osteoblasts communicate with one another via cytoplasmic processes, which lengthen and become more prominent as the surrounding matrix matures. When they have completely encased themselves in bony lacunae, these cells are referred to as osteocytes, whose cytoplasmic extensions, housed in canaliculi, enable them to maintain contact with neighboring cells and to receive nutrients. Osteoclasts, the third cell type, are large, multinucleated, motile cells derived from the fusion of blood-derived monocytes. They are present in areas of bone undergoing resorption, where they reside in depressions (Howship's lacunae) created by enzymatic matrix dissolution. Unlike osteoblasts, they have an acidophilic cytoplasm, and during periods of activity the cell membrane develops a ruffled border,

which increases the surface area available for enzymatic resorption. Consistent with their degradative function, the osteoclast's cytoplasm contains numerous free polysomes, mitochondria, and lysosomes. They secrete acid, collagenase, and other proteolytic enzymes that attack bone matrix and liberate calcified ground substance (Junqueira et al., 1992).

The various activities of these different bone cell types are coordinated in the daily process of bone remodeling, in which the skeleton responds and adapts to mechanical stress. The relationship between applied load and bone morphology is embodied in Wolff's "Law of Bone Transformation", which states: "Every change in the function of a bone is followed by certain definite changes in internal architecture and external conformation in accordance with mathematical laws" (Wolff, 1892). In other words, form follows function. The process by which mechanotransduction, or the transformation of a mechanical signal into a cellular response, occurs is the subject of a broad range of research efforts, with many of the details still to be elucidated. However, the basic mechanism is thought to involve the activation of a coupled response between osteoblasts and osteoclasts (Frost, 1987). It is through coordination of the processes of bone resorption and deposition, on both a cellular and a gross structural level, that bone architecture and density may be modulated in response to mechanical stimuli.

BACKGROUND

The effects of mechanical strain on skeletal tissues have been shown to induce both structural and genetic responses. Bone is perhaps the pre-eminent example of the close relationship between structure and biological function, in that its constituent cells are required to transduce mechanical signals into a physiologic response. The processes of bone growth and

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remodeling have been shown to involve the coordinated activities of various structural elements within bone (*i.e.* bone acting as an organ), as well as an equally complicated cellular response requiring fibroblasts, osteoblasts, and osteoclasts to act in concert with other cells to effect macroscopic changes. Studies involving the externally-loaded avian ulna model (Rubin and Lanyon, 1985) have demonstrated a direct correlation between strain magnitude due to differential loading patterns and compensatory changes in bone mass. On a cellular level, mechanical stimuli have been shown to effect a genetic response in osteoblasts through a variety of molecular mechanisms. This response includes ion fluctuations, changes in second messenger pathways, and variations in gene expression, resulting in altered expression of proteins and other signalling molecules, which in turn modulates cellular activity.

One such protein which appears to play a critical role in bone growth and remodeling is osteopontin (OPN), one of the predominant non-collagenous proteins found in the extracellular matrix of bone (Gerstenfeld et al., 1990, Gotoh et al., 1990). Osteopontin is a very acidic ~66 kDa phosphorylated glycoprotein composed of approximately 300 amino acids. A number of conserved functional domains have been discovered within its primary structure, which include several protein kinase recognition sites (Salih et al., 1995), recognition sites for N- and O-linked glycosylation, a poly-aspartate domain, and an RGD (Arg-Gly-Asp) recognition site responsible for integrin-mediated cell adhesion (Peirschbacher and Ruoslahti, 1984; Somerman et al., 1987) . Evidence of a chemotactic domain near the carboxyl terminal of OPN has also been found, and this portion of the molecule is thought to interact with a variant form of the CD44 receptor (Weber et al., 1996). The ability of osteopontin to interact with integrin and CD44 receptors has served as the basis for a number of research efforts concerning osteopontin's role in cell-

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matrix interactions and cellular migration (Liaw et al., 1995; Weber et al., 1996).

Osteopontin accumulates in the bone matrix of a wide variety of vertebrates, and it is expressed in many non-skeletal tissues as well. It has been shown to be an early response gene during T-cell (Patarca et al., 1989) and macrophage (Singh et al., 1991) activation, as well as an immediate early response gene to protein kinase C activation (Smith et al., 1987; Moore et al., 1991; Rafidi et al., 1994). Osteopontin has also been identified as a serum marker of malignant cell growth and has been found within metastatic tumors from a variety of primary cell origins (Senger et al., 1989). Lastly, mechanical stimulation has been shown to induce osteopontin expression in bone cells, including fibroblasts, osteoblasts, and osteoclasts (Harter et al., 1995; Kubota et al., 1993; Toma et al., 1997).

The identification of osteopontin's integrin-binding domain has fueled numerous studies aimed at discovering its role in adherence and migration of osteoblastic (Gotoh et al., 1990; Oldberg et al., 1988), fibroblastic (Sauk et al., 1990; Somerman et al., 1987), and osteoclastic (Flores et al., 1992; Ross et al., 1993) cell types. Integrins are a family of cell-surface receptors that mediate attachment of cells to the extracellular matrix, as well as functioning in cell-cell adhesive interactions. They are thus involved in regulation of embryonic development, tumor cell growth and metastasis, leukocyte homing and activation, apoptosis, and the cellular response to mechanical stress. The receptors are transmembrane heterodimers composed of alpha and beta subunits, whose structural complexity is increased via alternative splicing (Hynes, 1992). In addition to their ability to bind to extracellular matrix components (*e.g.* fibronectin, collagen, vitronectin), certain integrins are also able to interact with soluble ligands (*e.g.* fibrinogen) or bind to counter-

receptors (*e.g.* ICAMs) on other cells. The latter situation is responsible for clustering, in which ligands bind to adjacent integrin receptors on the cell surface, and the formation of focal adhesions, in which these aggregates are linked to actin filaments and intracellular cytoskeletal complexes. These adhesion plaques contain a variety of proteins, both structural (*e.g.* vinculin, talin) and regulatory (*e.g.* tyrosine kinases, such as focal adhesion kinase), as well as protein kinase C, the proteolytic enzyme calpain II, and several phosphoproteins (Jaken et al., 1989; Schaller et al., 1992; Burridge et al., 1992; Turner, 1991). The evidence points to beta-1 and beta-3 integrins' cytoplasmic domains as key determinants of focal adhesion localization (Wayner et al., 1991), while the alpha-subunit is also thought to play a role, via interaction with the beta-subunit, in inhibiting localization of unoccupied integrins (Ylanne et al., 1993).

Aside from their role as structural receptors that link the cytoskeleton to the extracellular matrix, integrins also function as transducers for two types of signals. The first is transmission of signals from the matrix into the cell. Unlike growth factor receptors, integrin cytoplasmic domains possess neither kinase nor phosphatase activities, but instead interact directly or indirectly with initiators of signaling cascades. This type of outside-in signaling can be induced by antibodies that cross-link receptors resulting in an increase in pH (Schwartz et al., 1991), phosphorylation of focal adhesion kinase (Kornberg et al., 1992), and calcium fluxes through activation of the Na/H antiporter (Pelletier et al., 1992), all of which occur in parallel with cell spreading. The second mode of signal transmission is initiated by the cytoplasm, with conformational changes in the integrin heterodimer propagated to the extracellular ligand-binding domain. This type of "inside-out" signaling has been shown, for example, to modulate the affinity of the fibrinogen receptor

(Shattil et al., 1987), adhesion of mouse NIH 3T3 cells to fibronectin and laminin substrates (Hayashi et al., 1990), and binding of COS cells to ICAM-1 (Hibbs et al., 1991). While it seems likely that cytoplasmic stimuli propagate through the membrane-spanning region of the integrin molecule, it has not yet been determined whether this process involves direct interactions between the alpha- and beta-subunit cytoplasmic domains or cytoplasmic/cytoskeletal molecules (Sastry et al., 1993).

It has been shown that osteopontin specifically binds to the alpha-v-beta-3 integrin isotype (Ross et al., 1993; Reinholt et al., 1990), which mediates cellular adherence and spreading. Other research has found that the alpha-v-beta-5 and alpha-v-beta-1 integrins can facilitate adherence and spreading of aortic smooth muscle cells on osteopontin substrates, but that cells deficient in the alpha-v-beta-3 isotype are incapable of migrating toward osteopontin (Liaw et al., 1995). Studies have also shown that osteopontin can induce CD44-dependent chemotaxis, thus providing a mechanism by which activated lymphocytes and monocytes migrate out of the bloodstream into sites of inflammation (Weber et al., 1996). The fact that metastatic tumor cells have been found to secrete markedly elevated levels of osteopontin (Senger et al., 1989) is evidence of its role in mediating cell-matrix interactions in a variety of cell types.

Osteoblasts are thought to play a crucial role in skeletal remodeling and repair through their ability to produce type I collagen, the major extracellular matrix protein of bone and a scaffold for mineral deposition (Glimcher, 1984), as well as their ability to adhere to and migrate along this bone matrix via integrin receptor interactions. The development of an embryonic chicken calvarial osteoblast system, in which the cells are able to differentiate and produce a calcified matrix (Gerstenfeld et al., 1987;

Gerstenfeld et al., 1988; Gerstenfeld et al., 1990), has made it possible to conduct *in vitro* studies on the nature of the osteoblast's role in bone development and maintenance. It has been shown that collagen deposition is controlled at the post-translational level and that collagen fibril formation and assembly of a collagen scaffold is possible in culture. The process of bone mineralization may also be studied *in vitro*, since foci of hydroxyapatite deposition may be induced in cultured osteoblasts. Investigations into the genetic basis for the activation of various osteoblastic genes, such as osteopontin, in response to mechanical perturbation is another area of research for which this system may be used because the strain parameters can be easily controlled.

Previous efforts to characterize the response of cultured osteoblasts to mechanical strain have concentrated on cell proliferation, changes in cell phenotype, and the production of second messenger molecules. Increased DNA synthesis (Brighton et al., 1991; Hasegawa et al., 1985; Neidlinger-Wilke et al., 1994), prostaglandin E_2 (PGE_2) production (Somjen et al., 1980; Yeh et al., 1984), activation of the phosphoinositide pathway (Sandy et al., 1989; Carvalho et al., 1994), and decreases in alkaline phosphatase activity (Brighton et al., 1991) in response to mechanical strain have all been observed within various osteoblast cell model systems. Many of these experiments have used strain-inducing devices consisting of cell-laden membranes, whose spatial patterns of deformation are either uniaxial or biaxial. While early systems involved tensile, uniaxial deformation of a rectangular membrane, more recent devices can induce biaxial membrane deformation, which generates a more uniform spatial strain pattern. The biaxial deformation of a circular membrane has been induced by vacuum, fluid injection beneath the membrane, and direct membrane displacement (Andersen et al., 1991; Banes

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et al., 1985; Banes et al., 1987; Belloli et al., 1991). The device used in this study (Diagram 1) is composed of a cassette containing independent cell culture wells which hold flexible-bottomed culture dish inserts. These membranes can be deformed vertically by platens controlled by asymmetric circular cams, which results in sinusoidal vertical displacement of the platens and creation of a spatially isotropic biaxial strain profile, as shown in Diagram 2 (Schaffer et al., 1994). ·

While it is clear that mechanical perturbation induces a variety of biological responses within osteoblasts, including up-regulation of osteopontin mRNA expression, the variability of the response suggests that more specific determination of the role of various strain parameters, such as duration and frequency, would be enlightening. Another factor that may play a significant role in the mechanotransduction process is the osteoblastic response to changes in fluid flow within the osteon. Studies have shown that flow-induced shear stress is associated with down-regulation of alkaline phosphatase, augmented prostaglandin E_2 production, and release of nitric oxide in osteoblastic cultures (Hillsley et al., 1997; Reich et al., 1993; Johnson et al., 1996; Owan et al., 1997), which suggests that flow may be an important mediator of the osteoblast's response to strain. While the integrin-based hypothesis involves receptor deformation as the basis for activation, the fluid flow model is based on changing ionic currents (streaming potentials) across/along the osteoblast membrane as the stimulus for cellular activation.

Fluid flow in bone originates from the leakage of plasma out of venous sinusoids into the bone marrow space. A transmural pressure gradient drives this fluid radially outward, resulting in a relatively steady flow from the vascular system to the lymphatic drainage system at the periosteal surface (Anderson, 1960). W.G. Seliger (1970) observed the presence of staining in

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the perivascular connective tissue of the vessels of Volkmann's canals in cat tibiae following injection of Thorotrast (colloidal thorium dioxide) label into the marrow cavity. In experiments with peroxidase tracers, Tanaka and Sakano (1985) have detected the presence of bone fluid transport pathways in the lacunar and canalicular spaces around bone cells and speculated on the presence of accessory routes. R.J. Montgomery et al. (1988) have also found evidence for interstitial fluid flow in the extravascular compartment of cortical bone. Using a ferritin marker injected into the nutrient artery of canine tibiae, ferritin rings were observed around haversian canals and more specifically within non-endothelialized branching channels in the perivascular matrix surrounding osteocytes. The observation of a heterogeneous pattern in which some capillaries retained ferritin for up to 60 min., while others transported the marker rapidly was attributed to a continuous cycle of capillary opening and closure via a sphincter at the arterial end. It was concluded that interstitial fluid is formed via filtration at the arterial end of the capillary, from which point it flows extravascularly through the osteon and is eventually either reabsorbed by the venous end of capillaries in adjacent haversian systems, or flows within a nonendothelialized channel until it reaches the periosteal lymphatic system.

The porous nature of bone and its compressibility are responsible for the superimposition of pulsatile flow onto steady flow during skeletal loading, and many studies have been conducted to elucidate the possible role of interstitial fluid movement as a mediator of mechanically-induced bone remodelling. These investigations have generally been based on studies which have shown that bone deformation generates electrical potentials. As early as 1955, Yasuda et al. detected electrical potentials generated between two rods implanted in long bone specimens, and found that they were negative in

compression areas and positive in tension areas. They then induced the formation of an "electric callus" in the periosteum by passing a 1 micro-ampere current through the femur for 3 consecutive weeks. From this they hypothesized that "the dynamic energy exerted upon bones is transformed into electrical energy, and the latter plays an important part in callus formation." Shamos et al. (1963) performed similar experiments using dry whole bones subjected to bending and compression and found that the sudden application of a static force resulted in a potential difference proportional to the stress. This phenomenon was initially called the "piezoelectric effect", in which electric polarization was found to be directly proportional to the stress placed on a bone. A number of investigators have hypothesized that this phenomenon arises either from the organic collagen component of bone, or from movement of hydroxyapatite crystals along collagen fibers as a result of shear stress (Shamos and Lavine, 1967; Anderson and Eriksson, 1970: Marino and Becker, 1971). Other studies, involving various wet-bone models to simulate the effect of bone bending *in vivo* , have attributed the observed electromechanical effect to the generation of streaming potentials, which are created by the movement of a polar fluid over a charged surface, as would result from the forced flow of an electrolyte through porous tissue during bone flexion. Johnson et al. (1982) devised a mathematical model for this phenomenon based on Darcy's Law, which relates the volume flow rate per unit area directly to fluid pressure and inversely to viscosity, and on Poiseuille flow , a similar model for fluid flow in a tube, representing an osteocytic canaliculus. Gross and Williams (1982) also espoused this model, and their *in vitro* bone bending studies have shown that electrical signal production was dependent on fluid flow through the bone specimen.

Subsequent studies attempted to localize the electrokinetic effect of streaming potentials to a particular histologic bony structure. Salzstein and Pollack (1987) developed an electrokinetic model for cortical bone based on low-frequency stress-generated potentials resulting from laminar flow through the tortuous microporosities surrounding hydroxyapatite crystals. Further support for this model has come from Scott and Korostoff's (1990) Instron testing of human and bovine bone samples under a wide range of strain frequencies (0.5-100 Hz) and using permeating fluids of varying conductivity and viscosity. A high correlation was shown between oscillatory strain magnitude and phase, as well as a linear relationship between the step response stress relaxation mechanism and the viscosity of the permeating fluid. They concluded that the streaming potential mechanism outlined by Salzstein and Pollack (1987) could account for the majority of electrical potentials generated in bone.

Other studies have focused on fluid pressure phenomena within the canaliculus as critical determinants of the response to skeletal loading. Petrov et al. (1989) devised a model to study the role of microstress concentration around the Haversian canal of an osteon during loading-induced fluid flux. Kufahl and Saha (1990) investigated the effect of various canaliculi diameters on the depth of penetration and magnitude of squeeze flow through these channels during loading. They concluded that a canalicular diameter of 0.2 um corresponds to a flow velocity adequate to nourish 4-5 concentric layers of an osteon. Experiments by Weinbaum et al. (1994) and Zeng et al. (1994), have taken this concept one step further by attributing the generation of streaming potentials to the flow of extracellular fluid across osteocytic processes within canaliculi, with flow rate determined by the fluid drag associated with the existence of a membrane

surface proteoglycan matrix. A comparison of *in vitro* and *in vivo* models by Otter et al. (1992) demonstrated differences in streaming potential magnitude and rate of rise between the two systems, thus indicating a need for further corroboration of theoretical models of fluid flow with *in vivo* studies.

While some studies have focused on the generation of an electromechanical response to loading, other investigations have centered around the effects of applied electric fields. For example, Shteyer et al. (1980) reported that subjecting fetal rat calvaria cells to pulsating currents *in vitro* resulted in increased DNA synthesis. The bone cell response to endocrine factors may also be mediated by electrical signals, as evidenced by the association of PTH binding with an increase in cAMP production and elevated cytoplasmic calcium ion concentration, which results in a long-term hyperpolarizing membrane potential response (Ferrier and Ward, 1986). Galvanotaxis studies by Ferrier et al. (1986) have shown that osteoclasts and osteoblasts migrate in opposite directions in response to a constant electrical field, with the former migrating towards the positive electrode and the latter toward the negative electrode. A theoretical model associating mechanical cell membrane deformation with the application of an external electrical field was devised by Bryant and Wolfe (1987), who demonstrated mathematically that the resulting cell lysis was due largely to mechanical surface tension produced in deforming the cell, rather than to the magnitude of the potential produced across the membrane. Several investigators have reported alterations in osteoblastic membrane potential in response to pH changes (Civitelli et al., 1987), as well as the existence of different cation-selective channels which are activated in response to mechanical stress (Davidson et al., 1990). Jeansonne et al. (1979) used injection dyes to show the rapid current-induced transfer of signals between osteoblasts, and

hypothesized that cell-cell junctions may facilitate calcium and/or endocrine movements between cells. A mathematical model devised by Harrigan and Hamilton (1993) to assess bone strain sensation by osteoblasts supports the idea that these cells sense mechanically-induced changes in transmembrane electric potential, and that the degree of cellular coupling influences bone's ability to respond to strain. Studies by Ozawa et al. (1989) of cultured bone cells subjected to a pulsing electric field have also shown evidence of increased DNA synthesis, but not of cAMP production. Furthermore, radioactive calcium ion uptake has been associated with DNA synthesis, which hypothesizes a role for calcium as a second messenger in the intracellular transmission of an electrical signal. Other support for this finding comes from whole-cell patch clamp studies in osteoblasts, which have confirmed the existence of mainly L-type calcium channels in the cell membrane (Grygorczyk et al., 1989). Williams et al. (1994) have also reported a transient increase in intracellular calcium due to laminar flow, but point out that the response is highly variable and asynchronous, especially at low shear stress levels.

The clinical implications of these findings have given birth to numerous research efforts directed at the treatment of various bone pathologies, including non-union of fractures and osteoporosis (Bassett, 1982). Using these studies as the basis for the treatment of osteoporosis of disuse, Martin and Gutman (1978) found that decreases in bone density resulting from limb immobilization of rat femurs were counteracted by the application of transcutaneous electrical fields (30 Hz, 100 V/cm for 2 or 8 h/day), and that the treated limbs were even larger that their original size due to increased bone mass. Capacitatively coupled electric fields have been shown to be effective in the treatment of recalcitrant non-union of long bone fractures

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in humans, irrespective of weight bearing (Brighton and Pollack, 1985). Similar phenomena have been observed in a feline model of orthodontic tooth movement (Davidovitch et al., 1980), in which alveolar bone resorption and the rate of tooth movement were enhanced in the presence of localized electric currents. Studies in canine tibiae by Otter et al. (1990) have related changes in intramedullary pressure, due to normal pulsatile blood flow, to the generation of transcortical streaming potentials *in vivo* in both in stressed (bending deformation) and unstressed bone. They concluded that while bone deformation may be responsible for an increase in the osteogenic response, pulse-related potentials may be the mechanism for baseline maintenance of bone mass.

AIMS

1. To study the effect of duration of mechanical strain on the expression of osteopontin by cultured embryonic chick calvarial osteoblasts *in vitro* .

2. To determine whether changes in the frequency of mechanical strain result in variations in osteopontin expression by cultured osteoblasts.

3. To investigate the role of fluid flow in the induction of osteopontin by osteoblasts subjected to variable levels of fluid-induced shear stress within the experimental system outlined below.

MATERIALS AND METHODS

Cell Culture System: An established embryonic chicken calvarial osteoblast system (Gerstenfeld et al., 1987; Gerstenfeld et al., 1988; Gerstenfeld et al., 1990) was used to evaluate the the genetic expression of cells on a flexible membrane. Osteoblasts were initially isolated by sequential

trypsin/collagenase treatment of 12-day embryonic chicken calvaria as described by Gerstenfeld et al. (1987). Cells found to express the osteoblastic phenotype were selected by initial incubation for two weeks in minimum essential medium (Sigma, St. Louis, MO) and grown in BGJ-b Fitton-Jackson medium (Sigma, St. Louis, MO) supplemented with antibiotics, 12.5 mg/mL ascorbate, and 10 mM glycerophosphate, while being maintained at 37 C in saturation humidity and 5% CO_2 in air. Culture medium was then changed every three days until cellular confluency was reached. The flexible membranes used in these experiments were composed of silastic or polyurethane polymers that were given a low energy gas plasma surface treatment, then sterilized and packaged (Becton Dickinson Labware, Franklin Lakes, NJ). Prior to cell inoculation, the membranes were coated with human fibronectin (Collaborative Biomedical Products, Bedford, MA) at a surface density of 20 ug/cm^2 following a modification of Ingber's procedure (Ingber, 1990). Cells were then plated on membrane stretch inserts at approximately 2.5×10^4 cells/cm^2. Control cells for the duration and fluid shear stress experiments were plated as described above and maintained for 8 hours in an unperturbed state in the same incubator as the stretch device.

Mechanical Perturbation: The mechanical perturbation device designed for these studies (Diagrams 1 and 2) delivered an experimentally-verified temporal and spatial displacement profile to a transparent elastomeric membrane (Schaffer et al., 1994). Due to manufacturing limitations, the silicone- based elastomer (Dow Corning Corp.) was replaced by a polyurethane-based membrane (Dow Chemical Corp., Midland, MI), for which the same strain pattern has been verified . Independent tests have shown that the same experimental conditions of strain magnitude and frequency produced homogeneous and isotropic strains (i.e. radial strain =
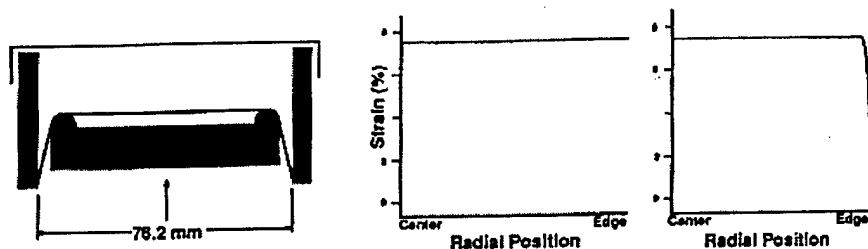
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Diagram 2. Platen indenter and membrane within the culture well. The circular membrane lies flat in the unstretched position and is constrained at its periphery by a rubber gasket stretched within the rim of the culture insert. The diameters of the circular platen and the stretch membrane are nearly the same, which results in radial and circumferential strains of equal magnitude, with magnitude dependent on the platen displacement (Schaffer et al., 1994).
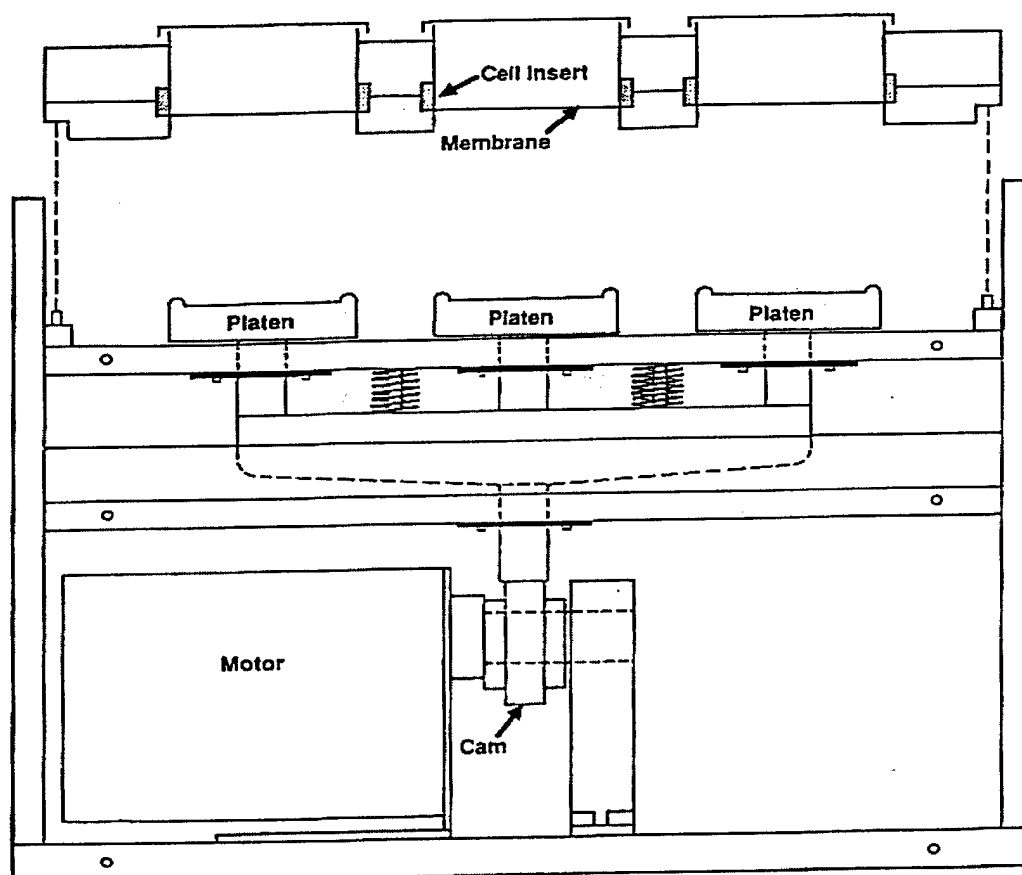

Diagram 1. Cross section of the cell deformation device through three of the six indenter platens and culture wells. The cassette, shown removed from the device, is holding flexible-bottomed culture inserts with deformable membranes that are secured peripherally. When the cassette is properly seated, the membrane contacts the platen (Schaffer et al., 1994).

circumferential strain = constant strain over the culture surface). To determine the effect of strain duration on osteopontin mRNA expression, osteoblasts were mechanically perturbed at a 1% uniform biaxial strain of constant frequency (0.25 Hz) for 1, 5, 15, 30 min. and 1, 2, 4, 8 hrs. Cells were then collected 8 hrs. after stretch initiation, similar to previous studies conducted in our lab by Toma et al. (1997), with an non-stretch control also collected after 8 hrs. of incubation in the same incubator as the stretch device. Triplicate membranes were used for each time period and for the control. The time period inducing a maximum genomic response was determined via biochemical analysis and served as the basis for the next set of experiments. .

In the second part of the study, cells were stretched for 8 hours (corresponding to the maximum duration response elicited) at frequencies of 0.1, 0.25, 0.5, and 1 Hz and collected immediately thereafter. Triplicate membranes were again used for each frequency. The purpose of these experiments was to determine whether changes in frequency induced a similar response to changes in the duration of applied strain, not whether the presence/absence of strain induced a response. Therefore, a non-stretch control was not evaluated, but instead the 0.25 Hz frequency level used in the first set of experiments (and in previous experiments conducted in this lab) served as the baseline control.

Lastly, an investigation of the effects of fluid shear stress was carried out within the same mechanical perturbation system. Since shear stress has been shown (Schaffer et al., 1994) to increase as one moves from the center of the stretch membrane towards the periphery (i.e. the site of platen contact), a general comparison of centrally- vs. peripherally-located cells was performed to determine whether variations in fluid shear stress were

associated with variations in the induction of OPN. Osteoblasts were plated on membrane inserts and stretched, as described by Toma et al. (1997), for 2 hours at different frequencies (0.1, 0.25, 0.5, and 1 Hz), with duplicate inserts for each frequency level, and then collected 8 hours after stretch initiation. In this case, a non-stretch control was used so that comparisons could be made among cells experiencing high shear, low shear, and no shear stress. At collection, cells were divided into two groups using concentric circular punches (McMaster-Carr Supply Co., New Brunswick, NJ) of 1-5/8 inch and 2-1/4 inch diameters, which cut the area of the membrane within the platen confines into concentric circles of fairly equal surface area. The portion of the membrane between the platen rim and the wall of the culture well was excluded from collection, as the strain profile in this area is non-uniform (Schaffer et al., 1994), which would have made it impossible to differentiate the effects of variations in fluid flow from variations in stretch-induced strain. RNA isolation and analysis was then carried out for the high- and low-shear groups of cells as described below.

Isolation and Analysis of RNA: Total RNA was isolated using Tri-Reagent TM (Molecular Center, Cincinnati, OH) according to the manufacturer's instructions. RNA was then resolved via Northern Blotting on 1% agarose gels containing 2.2 M formaldehyde (Toma et al., 1997) with 5 ug of total RNA loaded per gel lane. A cDNA probe encoding the complete sequence of chicken osteopontin (Moore et al., 1991) was used to quantify levels of expressed osteopontin mRNA. Northern blotting with ^{32}P-labeled OPN cDNA was carried out at 65 C in 2.5 X SSC, 50 mM Na phosphate buffer, with 100 ug/ml single-stranded salmon sperm DNA, for 18 to 24 hours in a rotating hybridization oven (Robins Scientific, Sunnyvale, CA). Autoradiograms were then quantified using an LKB Ultra II scanning densitometer (LKB, Broma,

Sweden). Values were normalized to 18S ribosomal RNA by re-hybridization of each blot to a ^{32}P-labeled conserved nucleotide sequence probe of the 18S ribosomal subunit (Ambion Corp., Austin, TX).

Statistical Analysis: All analyses were performed at least three times and were repeated with three separate preparations of cells. Data was evaluated as a mean +/- 1 standard deviation. Statistical analysis for the strain duration and frequency experiments consisted of ANOVA (Analysis of Variance) to evaluate the significance of the overall data trend, as well as two-sample t-tests (with and without the Bonferroni correction method) to evaluate significant differences between specific pairs of data. For the frequency/flow experiments, a t-test for each high/low pair of results was conducted to determine whether a significant variation in the OPN response occurred at each frequency studied.

RESULTS

The effect of strain duration on the induction of osteopontin expression.

In the first set of experiments, cultured osteoblasts were subjected to strain induced by membrane stretch for periods of 1 minute up to 8 hours to assess the effect of stretch duration on OPN induction. The strain frequency and applied strain were kept constant at 0.25 Hz and 1% respectively, as previously described in experiments by Toma et al. (1997). While previous studies (Toma et al.,1997; Meazzini et al., 1998) have provided data for a 2-hour period of stretch, the aim of this part of the project was to assess the response to both shorter and longer periods of strain, to detect any pattern of OPN expression (*i.e.* linear vs. exponential response, peak response, plateau) , and to see if the variations between time points were significant. The

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following data (Table 1 and Figure 3) were collected via autoradiographic quantification for separate trials using three separate osteoblast preparations. Osteopontin expression is reported as a percentage of non-stretch control expression using calculations involving the integrated areas under the OPN and 18s (control) curves displayed by transillumination densitometry, which reflect their respective autoradiographic band densities. For each time period and for the control cells, OPN area/18s area was calculated to determine the normalized OPN density. Then each of these OPN density values was divided by the normalized non-stretch control density value and then multiplied by 100 to determine OPN expression as a percentage of control. Values greater than 100% indicate that OPN expression exceeded that of the non-stretch control . The mean and standard deviation were calculated for the three groups of cells (n=3). Analysis of Variance (ANOVA) was then performed to test for significance of the variation among the calculated averages for each time period. For an F-distribution with $(k - 1) = (8 - 1) = 7$ and $(n - k) = (24 - 8) = 16$ degrees of freedom, $0.001 < p < 0.005$. Therefore, the elevation in OPN expression corresponding to an increase in the duration of strain was highly significant at the 0.005 level.

1 m 5 m 15 m 30 m 1 h 2 h 4 h 8 h ctrl
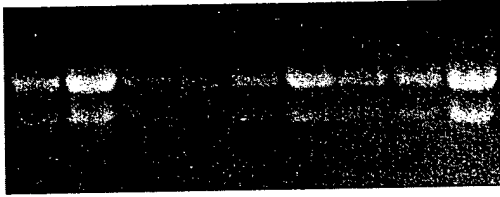
Representative
gel band image
for strain
duration
experiments.

OPN
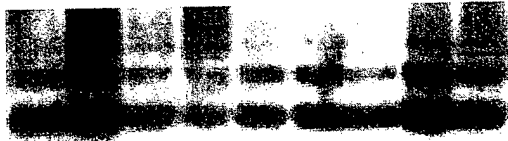
Autoradiogram
image.
Hybridization
with OPN cDNA
probe.

18S
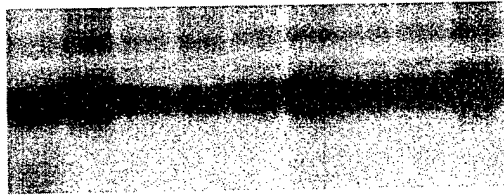
Autoradiogram
image.
Hybridization
with 18S
cDNA probe
(control).

Figure 3. The effect of strain duration on the OPN response. With the
exception of the 1 h. time point, a gradual increase in OPN expression
(expressed as % of controls) resulted from increasing duration of stretch-
induced strain. Error bars are +/- 1 standard deviation.

Table 1.   **Duration of Strain:**   **Osteopontin Expression as a Percentage of Control Expression**

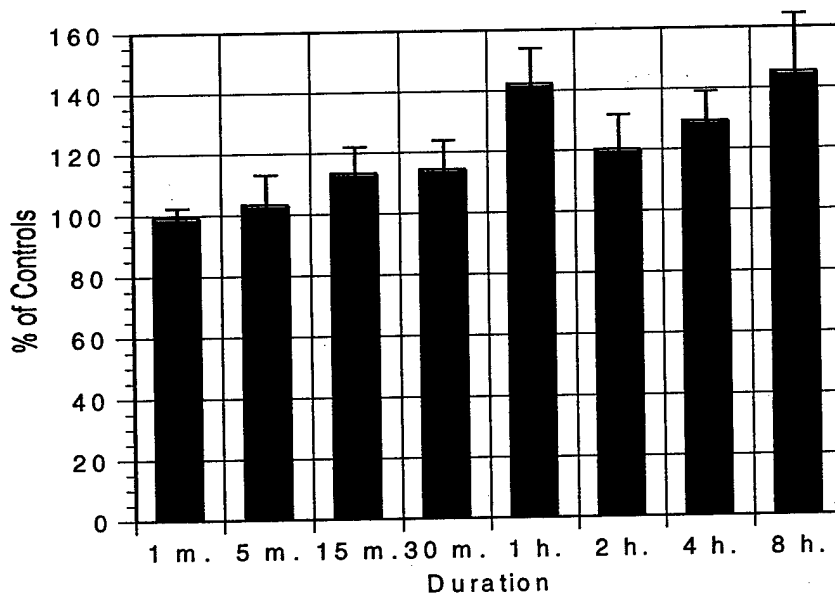| Trial #	*Duration (% of Controls)*							
	1 m	5 m	15 m	30 m	1 h	2 h	4 h	8 h
1	95	95	105	105	145	110	120	130
2	100	101	111	113	153	116	126	137
3	102	115	123	125	129	133	140	167
Mean (n=3)	99	103	113	114	142	120	129	145
Standard Deviation (+/-)	3.6	10.3	9.2	10.1	12.2	11.9	10.3	19.7

To determine where the significant differences occurred, a series of two-sample t-tests was conducted between subsequent pairs of strain duration values , as well as between more widely-spaced data pairs (Table 1a).   Similar t-tests were also performed using the more conservative Bonferroni correction modification, but no significant differences were found (for $p < 0.05$) in this case.

**Table 1a.** Two-sample t-tests to determine significant differences between pairs of strain duration values.

Duration pair	Test statistic (t_4)	Significance (p-value)
1 m/5 m	-0.63	$p > 0.20$
5 m/15 m	-1.25	$p > 0.20$
15 m/30 m	-0.13	$p > 0.20$
30 m/1 h	-3.06	$p < 0.05$
1 h/ 2 h	2.24	$p < 0.10$
2 h/4 h	-0.99	$p > 0.20$
4 h/ 8 h	-1.25	$p > 0.20$
1 m/8 h	-3.98	$p < 0.02$
1 m/1 h	-5.86	$p < 0.01$
2 h/8 h	-1.88	$p < 0.20$

The statistical values show that no significant differences (p-values > 0.20) occurred for strain durations of less than 1 hour. However, there was a significant degree of OPN activation between 30 m. and 1 h. ($p < 0.05$), 1 m. and 1 h. ($p < 0.01$), and 1 m. and 8 h. ($p < 0.02$). This might indicate that a certain minimal period of strain --in this case, between 30 minutes and 1 hour-- is required to induce OPN transcription at a frequency of 0.25 Hz. In other words, the existence of a strain duration threshold may be hypothesized.

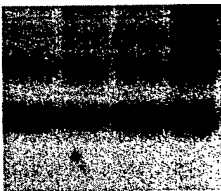*The effect of strain frequency on the osteopontin response*

The purpose of this portion of the study was to study the effect of variations in strain frequency on the OPN response. In order to isolate this parameter, the duration (8 hours) and magnitude (1%) of strain remained constant, while the frequency of membrane perturbation (0.1, 0.25, 0.5, 1 Hz) varied. The results of the duration experiments indicated that for the time range encompassed in the study, the greatest OPN response resulted from 8 hours of perturbation, so an 8-hour strain interval was chosen for all frequency determinations . Normalized values were calculated in the same manner as for the duration experiments, with frequencies for the three trials expressed as a percentage of control (Table 2 and Figure 4). In this section, the 0.25 Hz response was used as a baseline standard for frequency values above and below it, so its normalized value was 100% for each trial as well as for the mean, and the standard deviation was zero. The data indicate that OPN mRNA levels increase in conjunction with elevations in strain frequency. It is interesting to note as well that the response is more dramatic at higher frequencies (greater than 0.25 Hz) than at levels below that of the 0.25 Hz control, although the mean for the 0.1 Hz values was obviously skewed by the abnormally large response for the second trial. However, even elimination of this value (151%) from the calculation of the mean would not alter the pattern of a noticeably larger OPN response at higher frequencies.

0.1 0.25 0.5 1 Hz
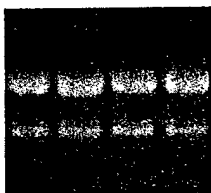
Representative
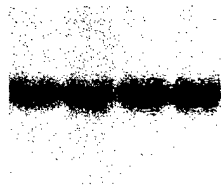gel band image
for strain
frequency
experiments.

OPN

Autoradiogram
image.
Hybridization
with OPN
cDNA probe.

18S

Autoradiogram
image.
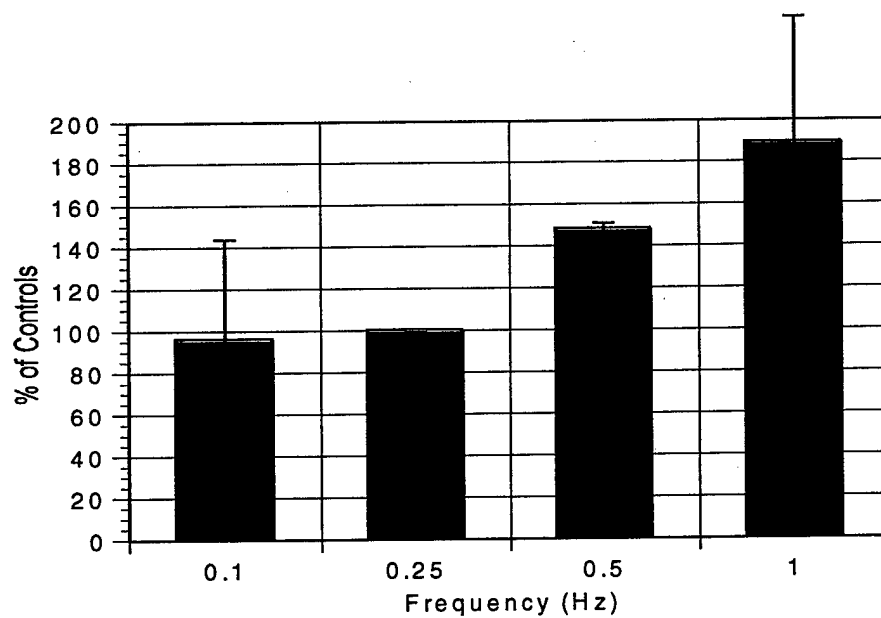Hybridization
with 18S cDNA
probe (control).

Figure 4. The effect of strain frequency on OPN expression. Increasing frequency
yields an increase in production of OPN mRNA (expressed as % of controls).
Error bars are +/- 1 standard deviation.

Table 2. Frequency of Strain: Osteopontin expression as a percentage of control expression

Frequency (% of Controls)

Trial #	0.1 Hz	0.25 Hz	0.5 Hz	1 Hz
1	63	100	150	256
2	151	100	149	174
3	73	100	144	138
Mean (n=3)	96	100	148	189
Standard Deviation (+/-)	48.2	0	3.2	60.5

The standard deviation values for the strain frequency determinations are much larger than those for the duration experiments, which would be expected to decrease and possibly eliminate the significance of the result. However, Analysis of Variance (ANOVA) demonstrated that for an F-distribution with k - 1 = 4 - 1 = 3 and n - k = 12 - 4 = 8 degrees of freedom, $0.05 < p < 0.1$. Therefore, we can reject the null hypothesis (*i.e.* variations in strain frequency do not cause a significant change in the OPN response) at the 0.1 level .

As for the duration experiments, more specific statistical analyses were conducted to determine where the significant differences occurred (Table 2a).

Table 2a. Two-sample t-tests to determine significant differences between pairs of strain frequency values.

Frequency pair (Hz)	Test statistic (t_4)	Significance (p-value)
0.1/0.25	-0.14	$p > 0.20$
0.25/0.5	-25.95	$p < 0.001$
0.5/1	-1.17	$p > 0.20$
0.1/0.5	-1.87	$p < 0.20$
0.1/1	-2.08	$p < 0.20$
0.25/1	-2.55	$p < 0.10$

From these values it may be deduced that variations in strain frequency within the 0.1 Hz to 1 Hz range may induce a highly significant OPN response (*i.e.* $p < 0.001$ for 0.25 Hz vs. 0.5 Hz). However, performing multiple t-tests increases the chances of committing a type I error, or rejecting the null hypothesis when it is true (Pagano and Gauvreau, 1993, p. 263). Therefore, a more conservative analysis using the Bonferroni correction modification was also carried out, which uses a pooled estimate of the common variance from all 4 groups, rather than relying on the data from one pair of samples. In this case, significant elevations in OPN expression were also found between 0.1 Hz and 1 Hz ($p < 0.02$), and between 0.25 Hz and 1 Hz ($p < 0.05$). This implies that variations in strain frequency below the 1 Hz level may have a significant effect on the OPN response, and that a frequency "threshold" may exist somewhere between the 0.25 and 0.5 Hz level, which triggers a significant elevation in gene expression.

The effect of flow-induced shear stress on the OPN response.

The purpose of this portion of the study was to detect any change in osteopontin expression resulting from differences in flow-induced shear stress experienced by osteoblasts residing on peripheral (higher shear) vs. central (lower shear) areas of the stretch membrane, and whether this was a frequency-related phenomenon. Results were obtained for both groups of cells (high and low shear stress) in three separate cell cultures and normalized as described above (Table 3 and Figure 5). For these experiments, a non-stretch control was used to rule out any unforeseen non-strain related positional effects experienced by cells in different areas of the membrane. As was the case in the second part of the study, the mean values for the three trials show an increase in OPN induction with increasing strain frequency. However, a clear and unexpected trend was also present, namely that peripherally-located osteoblasts produced lower levels of OPN mRNA compared to cells in the center of the membrane at each strain frequency studied.

	0.1 Hz		0.25 Hz		0.5 Hz		1 Hz		ctrl	
	P	C	P	C	P	C	P	C	P	C

Representative gel band image for flow-induced shear stress experiments.

OPN

Autoradiogram image. Hybridization with OPN cDNA probe.

18S

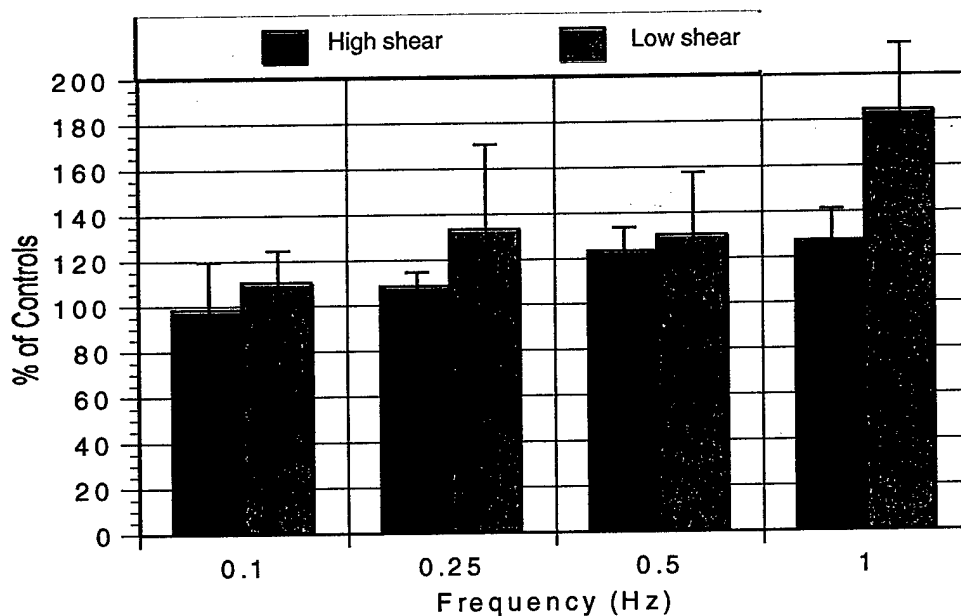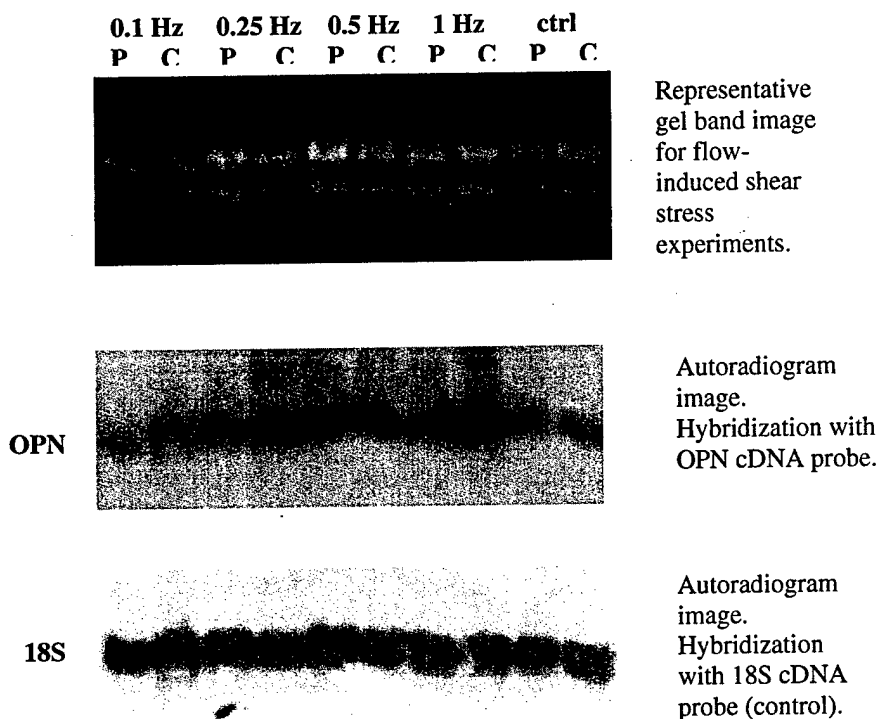Autoradiogram image. Hybridization with 18S cDNA probe (control).

Figure 5. Effect of fluid shear stress on cells located peripherally (high shear) and centrally (low shear) on the stretch membrane. For all frequencies, peripheral cells produced lower levels of OPN mRNA (expressed as % of controls) than those located in the center of the membrane. Error bars are +/- 1 standard deviation.

Table 3. **Effects of strain frequency and high *vs.* low fluid shear stress**

Frequency (Hz)/Flow rate (H/L) - % of Controls

Trial #	0.1 Hz P	0.1 Hz C	0.25 Hz P	0.25 Hz C	0.5 Hz P	0.5 Hz C	1 Hz P	1 Hz C
1	114	119	113	123	110	110	111	170
2	107	92	100	100	132	46*	139	164
3	73	118	111	175	126	150	132	219
Mean (n=3)	98	109	108	132	122	130*	127	184
Standard Deviation (+/-)	21.9	15.3	7	38.4	11.4	28.3*	14.6	30.2

P = peripherally-located cells;   C = centrally-located cells

* This value is abnormally small and has been excluded from calculations of the mean and standard deviation.

To determine whether this unexpected pattern was statistically significant or the result of chance error, a 2-sample t-test for each frequency pair was conducted, and the results are shown in Table 3a.

Table 3a. Two-sample t-tests comparing high and low fluid shear data for each frequency.

Frequency (Hz)	Test Statistic (t_4)	Significance (p-value)
0.1	- 0.71	$p > 0.20$
0.25	- 1.10	$p > 0.05$
0.5	- 0.425	$p > 0.05$
1	- 2.94	$p < 0.05$

This analysis indicates that the difference in OPN induction between the two groups of cells (central and peripheral) was significant only at higher frequency levels ($p < 0.05$ at 1 Hz). What remains to be answered is whether some unforeseen mechanical factor(s) is/are responsible for this result, whether this pattern of gene expression reflects more complex wave interactions within the device than had been originally hypothesized, or whether there is a flaw in the experimental design of this part of the study.

DISCUSSION

The experiments discussed above have been designed to show the effect of various physical parameters on the strain-induced production of osteopontin by chick calvarial osteoblasts *in vitro* . The osteopontin response obtained in this study further validates the membrane stretch model used in our lab and in others (Hasegawa et al., 1985; Gilbert et al., 1989; Brighton et al., 1991; Toma et al., 1997; Meazzini et al., 1998) for mechanically-induced strain of osteoblasts *in vitro*. These results suggest that changes in the duration and frequency of strain, as well as in the magnitude of flow-induced shear stress,

induce variations in osteopontin expression and present further evidence that OPN is a mechanically responsive gene.

The study of strain duration encompassing time periods of 1 minute to 8 hours resulted in a gradual elevation of the OPN response with increasing duration of strain. There does not appear to be any levelling off of OPN production within this time span, nor is a peak response evident followed by a decline. Previous experiments by Toma et al. (1997) have examined the time course for OPN induction in osteoblasts perturbed for 2 hours and detected a peak response at 9 hours post stretch initiation followed by a decline. This study, on the other hand, aimed to determine whether strain duration itself causes a significant increase in osteopontin expression, and the data suggest that it does. Experiments by Toma et al. (1997) also indicate that the time required for OPN transcription to reach peak levels is significant and occurs well after mechanical strain has ceased, which suggests that mechanically-induced strain continues its inductive effect long after the physical stimulus has been removed. In this study, strain durations shorter than 2 hours were used to see if there was some minimum time period required to induce an OPN response, and the results show that very short durations (e.g. 1 m. and 5 m.) produce almost the same response as the non-stretch control osteoblasts (99% and 103% of controls respectively), even at 8 hours post-stretch. It is only when the strain duration reaches 1 hour or more that any dramatic increase in the response occurs, which suggests that a certain time-related strain threshold may exist. Alternatively, a short duration of strain may induce a concomitant short duration of mRNA production, which peaks and then declines before the cells are collected at 8 hours.

The fact that the response after 8 hours of perturbation with immediate collection was almost 1.5 times that after 1 minute of strain with an 8-hour

delay in collection indicates that the final magnitude of the response is indeed related to the absolute strain duration, rather than to the strain application itself. If the post-strain time to collection were the more significant determinant of an OPN response, then the shorter durations (e.g. 1 m. and 5 m.) should have induced responses that were significantly greater than the control response at 8 hours (*i.e.* greater than 100%). Since Toma et al. (1997) have shown a peak response occurring as long as 9 hours post stretch initiation, it would be logical to conclude that if the response were determined by the length of this period, then the cells stretched for 1 minute and those stretched for 8 hours would have the same response, since they were both collected 8 hours after strain began. However, our data indicate that the longer duration cells (*i.e.* greater than 1 hour stretch), which had a shorter post-stretch rest period, produced significantly greater levels of OPN compared to cells experiencing a shorter duration of strain and a long rest period. These results therefore support the hypothesis that a strain duration threshold exists, most likely around 1-hour, since significant elevations in OPN expression were not detected for strain durations below this point. A maximum threshold does not seem to exist within this range, since the data show a continual increase with no evidence of a peak or plateau phase. However, the possibility exists that such a phenomenon may occur as a result of strain durations longer than 8 hours.

Meazzini et al. (1998) used a similar protocol in which cells were stretched for a limited time period (2 hours) for four consecutive days and found that the OPN response was down-regulated, from which a chronic adaptation mechanism was hypothesized. This study, on the other hand, examined a longer periods of strain (up to 8 hours), but for one period of activation only. Therefore, it may be that the time course of the strain (*i.e.* interrupted vs.

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continuous) determines the maximal limit of the OPN response, whereas the duration of mechanical stimulation may control the magnitude of OPN expression for a single period of strain. The gradual increase in OPN induction within the range encompassed in this study also does not suggest an all-or-none phenomenon as seen in neuronal impulse transmission, but instead denotes a gradual, fairly linear response, with the exception, perhaps, of the 1-hour time point. Therefore, the nature of the duration-related osteopontin response induced in cultured osteoblasts appears to be fairly linear, to have an induction threshold at about 1 hour, and to increase continuously with strain duration for up to 8 hours and possibly beyond. Further studies of longer strain duration periods would verify the existence of possible plateau or peak/decline phenomena.

The results of the frequency experiments indicate a significant increase in the osteopontin response with increasing frequency when compared to the 0.25 Hz baseline used in previous studies (Toma et al., 1997; Meazzini et al., 1998). For frequencies less than 0.25 Hz (*i.e.* 0.1 Hz), the average response was less than 100% (although this was marginal), whereas OPN mRNA production almost doubled (189% of control) at the 1 Hz level. For this group of experiments, the incremental increase in OPN expression was much greater, particularly at the higher end of the frequency range, than that for the duration portion of the study, although a 1:1 correlation was not observed (*i.e.* doubling the frequency did not result in doubling of the OPN response). The p-value calculated for ANOVA analysis of this data set was greater than that for the duration experiments, which is probably related to the large standard deviations for the 0.1 Hz and 1 Hz groups. A more powerful study with a larger sample size would undoubtedly increase the significance of these results. However, in spite of the greater variability and relatively

small sample size (n=3), the data do suggest that variations in strain frequency play a significant role in stimulating OPN expression.

As with the strain duration portion of the study, no plateau or peak/decline trend in OPN expression was observed for the given frequency range (0.1 - 1 Hz). It has been estimated that the frequency range experienced during physiological bone loading is between 0.1 and 20 Hz (Harrigan and Hamilton, 1993), with lower frequencies (1-2 Hz) corresponding to locomotion and higher ones (15-20 Hz) associated with maintenance of posture (Weinbaum et al., 1994). Assuming a 1:1 strain transmission between the bone and its component cells, the range examined in this study would correspond to loading frequencies experienced during locomotion, which suggests that frequencies at the lower end of the physiologic range can result in significant osteoblastic activation and that osteopontin plays a role in this response. Further experiments investigating the effect on OPN induction at higher frequencies (*e.g.* 10-20 Hz) might elucidate the role, if any, of osteopontin in postural control phenomena, as well as uncover any limit to its frequency-related induction.

The demonstration that osteopontin expression can be induced by increases in the duration and frequency of strain can be placed in the context of integrin-related osteoblastic activation. It has been shown that fibronectin is up-regulated in response to mechanical perturbation (Schaffer et al., 1996) and that fibronectin binding alone induces OPN expression (Carvalho et al., 1998). Carvalho et al. (1998) have also found that fibronectin ligation by osteoblastic surface integrins induces up-regulation of the OPN gene, whereas osteoblast attachment to a non-integrin-binding (non-receptor-mediated) substrate, such as tissue culture plastic, does not. This suggests that different mechanisms and surface molecules may mediate cell

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attachment and integrin binding. Meazzini et al. (1998) have found that osteoblasts respond to stretch-induced mechanical strain by the elongation and rearrangement of cytoskeletal fibers, actin polymerization, and microtubule reorganization. Furthermore, vinculin, a component protein of focal adhesion complexes, was also shown to be up-regulated in response to strain, which indicates a role for integrin-mediated activation of intracellular signalling cascades resulting in a genomic response. Formation of focal adhesion complexes has been shown by a number of investigators (Mueller et al., 1989; Miyamoto et al., 1995) to be associated with integrin binding to a substrate. However, Miyamoto et al. (1995) have found that integrin clustering alone, in the absence of specific ligand-receptor interactions, is insufficient to induce OPN gene expression. Therefore, integrin receptor occupancy via specific ligand binding appears to be a critical step in the mechanotransduction process, and explanations for our results and for those of other related studies must account for this. Since fibronectin production is also up-regulated by mechanical strain, and since it is known to bind specifically to integrin receptors, a mechanism can be hypothesized whereby increases in the duration and frequency of continuous strain result in increased fibronectin production and binding to integrin receptors. Such a mechanism would be expected to have a dose-response relationship, in which the number of occupied receptors would correlate with the magnitude of the response and 100% receptor occupancy would correspond to a maximum limit. Since these experiments did not detect an upper limit to the OPN response, further studies encompassing a broader range of strain duration and frequency variables are indicated.

Another explanation for the results obtained in this study might be that the mechanical stimulus being sensed by cultured osteoblasts (and

presumably in bone) is related to shear stress caused by fluid flow along the cell membrane. The mechanism for this type of signal transduction may be related to the generation of streaming potentials along the membrane surface, which in turn activate ion channels, resulting in elevated intracellular calcium levels and subsequent activation of various second messenger pathways (Harrigan and Hamilton, 1993; Weinbaum et al., 1994; Williams et al., 1994). Alternatively, the pressure gradients within bone canaliculi, which fluctuate during loading as interstitial fluid is forced in and out of the extracellular spaces (Kufahl and Saha, 1990), might induce physical deformation of the cell membrane and cytoskeletal elements in a manner that is comparable to stretch-induced strain.

In an attempt to discern whether or not fluid flow plays a role in OPN induction, cells experiencing greater fluid shear stress were separated from those experiencing lower levels by dividing the stretch membrane into concentric central (lower shear) and peripheral (higher shear) circular regions, and then quantifying the genetic response to see if there was a significant difference between the two groups at different frequency levels. Surprisingly, for every frequency level, OPN induction was greater in the centrally-located cells (lower shear) than in the peripheral ones (higher shear). Furthermore, t-tests conducted for each pair of results (higher vs. lower shear) indicated that only at the upper end of the frequency range($i.e.$ 1 Hz) was there a significant difference ($p < 0.05$) between the two groups. Other investigations of the effect of fluid shear stress on bone cells in $vitro$ have found increases in intracellular calcium ion concentration (Williams et al., 1994), as well as elevated prostaglandin E_2 and inositol trisphosphate levels (Reich and Frangos, 1993) with increasing shear stress. In a study concerning the effect of fluid shear stress on cultured rat calvaria

osteoblasts, Hillsley and Frangos (1997) reported no change in collagen or osteopontin expression after 8 hours of pulsatile flow. However, experiments by Owan et al. (1997) using cultured mouse osteoblasts subjected to four-point bending at various loading rates, found that a 3- to 4-fold increase in OPN expression was associated with higher displacement rates, which is similar to our findings. Their study also tried to determine the effect of fluid flow-associated strain as the culture plate moved through the medium by varying strain magnitude and displacement rate of the plate independently. The findings that variations in strain magnitude and rate had no effect on OPN expression, while larger displacement rates, associated with greater fluid forces, yielded a significant increase supports the hypothesis that flow-induced shear stress does indeed play a role in OPN induction.

The results of the frequency/flow portion of this study correspond in several ways to those obtained by Owan et al. (1997). The results shown here also provide evidence of a significant increase in OPN expression with increasing duration of strain, as well as a significant modulation of the OPN response in cells subjected to different levels fluid shear stress at higher frequencies (i.e. 1 Hz). This study also detected frequency-related induction of the OPN response for strain levels at the low end of the physiologic range. Therefore, it is possible that the biaxial membrane stretch model with a sinusoidal wave form produces a significantly different genetic response from the four-point bending model with a triangular wave form used in the study by Owan et al. (1997). Since bone deformation experiments, including those using a four-point bending apparatus, are traditionally conducted using either a step-load or a sinusoidal deformation mode (Salzstein et al., 1987), the mechanical device used in this study may more accurately represent the loading situation found *in vivo*. However, Owan's experiments have the

advantage of reporting strain levels measured directly via attachment of a gauge to the bottom of the culture plate, which would not be possible in the membrane stretch apparatus. Therefore, one disadvantage in using the biaxial membrane stretch model is that actual strain values cannot be easily measured,. Furthermore, the differences in the flow rates experienced by peripherally- vs. centrally-located cells cannot be easily calculated. Whereas most of the fluid flow experiments to date have been conducted under conditions of uni-directional, laminar flow, the membrane stretch model used here produces radial flow with interference patterns generated by interactions between incident waves and those reflected off of the circular rim of the tissue culture insert. In order to accurately determine the flow rates present in different areas of the membrane and then to convert these into membrane surface strain values experienced by the two groups of cells isolated in the third part of the study, it may be helpful to engineer a computer model to further analyze this system.

One possible explanation for the inverse relationship between the level of shear stress and OPN expression, is that at higher frequencies (*i.e.* 1 Hz) the peripheral cells experience so much strain that the OPN response is down-regulated. This would be analogous to the conduction phenomenon in which neurons that are over-stimulated will fail to respond until the membrane potential has had time to recover. While the same trend was apparent for frequencies less than 1 Hz, the individual t-tests indicate that the differences between central and peripheral OPN expression are not statistically significant, so there may be no appreciable difference between the two groups of cells. Furthermore, a variety of effects, including frequency and duration of strain, magnitude of strain, elasticity of the membrane, and viscosity of the culture media, might combine in unforeseen ways to produce

this unexpected result. It may also be hypothesized that fluid shear stress plays a greater role at certain frequency levels, while physical deformation may dominate at others. Similar studies to examine this phenomenon at strain frequencies greater than 1 Hz would help to confirm this trend, as well as give a more comprehensive picture of the role of fluid flow in stimulating a response within this particular system.

In any case, assuming that the strain is indeed uniform within the confines of the platen circumference, significant differences in the OPN response between central and peripheral osteoblasts at higher frequencies suggests that flow-induced shear stress does have an inductive function. The results of the three sections of this study taken together indicate that both generalized cytoskeletal deformation and flow-induced membrane shear stress may play a significant role in the stimulation of osteopontin expression. They also show that even at lower frequencies (less than 1 Hz), significant increases in OPN mRNA were detected, while fluid flow exerted a significant effect only at the upper end of the experimental range. This suggests that different inductive mechanisms for OPN may operate within the same cell according to the applied strain frequency level. This study does not elucidate the differences between the two pathways in terms of a specific transduction mechanism, nor does it indicate where they might converge intracellularly, if at all. Resnick et al. (1993, 1995) have identified a shear stress response element (SSRE) in the promoter region of several endothelial cell genes responsive to flow-induced shear stress. However, it also has been pointed out (Meazzini et al., 1998) that shear stress resulting from fluid flow can itself deform the cell membrane along with various surface receptors, including integrins, especially if it is of sufficient magnitude. Streaming potentials may be incorporated into this theoretical mechanism as well,

either arising from flow-induced charge displacements, or in conjunction with integrin-mediated activation of membrane ion channels. Hence the combination of ion fluctuations, signalling cascade activation, and integrin clustering may be responsible for converting integrin receptor interactions into a genetic response . Therefore, these results suggest that numerous possible strain-related mechanisms might play a role in the induction of osteopontin, and that it may be the nature of the applied strain that determines which pathway, or combination of pathways, is selected to transduce the mechanical signal.

CONCLUSIONS

This investigation of the effects of strain duration, strain frequency, and flow-induced shear stress on the induction of osteopontin in cultured chick calvarial osteoblasts has yielded further insight into the physical mechanisms controlling expression of this gene. These results indicate that both the duration of mechanical perturbation, and the frequency of applied strain have significant positive effects on OPN induction with increases in frequency yielding up to a two-fold elevation in gene transcription versus control at the 1 Hz level. Increasing the duration of applied strain also produced a significant elevation in OPN mRNA levels, but the response was more gradual, reaching a maximal level of 145% of control following 8 hours of strain. Examination of the effect of fluid-induced shear stress on cultured osteoblasts revealed differences in OPN mRNA expression between cells located peripherally on the membrane compared to those in the center, but this was significant only at the 1 Hz, suggesting that a certain minimal flow rate is required to activate this particular mechanism of OPN induction. The unexpected finding of an association between cells thought to be

experiencing a higher rate of fluid shear with the induction of a lower OPN response (and vice versa) indicates that this model for fluid shear may be much less straight forward that was originally thought and that undetermined confounding factors (*e.g.* complex flow interference patterns) make a straight forward explanation of this result unlikely. A comprehensive interpretation of the results from all three portions of this study suggests that both integrin-controlled and flow-mediated pathways may play a role in the transduction of a mechanical stimulus into a genomic response, and that the nature of the stimulus may determine the predominance of a specific signaling pathway.

FUTURE STUDIES

While these experiments offer insight into the effects of changes in various strain parameters, their scope is somewhat limited, and further research would give a broader perspective on these forms of osteopontin induction. The duration studies, for example, show a fairly steady increase in OPN expression, but no levelling off or decline is evident. Extending the duration of strain to 24 hours or more might enable the upper limit of the OPN response to be defined, or possibly elucidate a change in the pattern of expression (*i.e.* differences in the response to short-term vs. long-term strain duration). Looking at this issue a different way, previous studies concerning the time course of OPN mRNA synthesis (Toma et al., 1997) following a 2-hour period of stretch could be expanded to see whether strain duration has an effect on the length of the peak/decline time course or on the magnitude of the peak response.

The same types of studies could be conducted for the frequency experiments. Since this thesis examined low-level strain frequencies, it

might be informative to examine how OPN expression changes in response to frequencies at the upper end of the physiological range (*e.g.* 15-25 Hz). These results may disclose the existence of a plateau in the frequency response, as well as enable comparisons to be made between various types of physical activity, their associated strain frequencies, and the role of OPN in the induced bone remodeling response. Time course studies for different frequency levels might also be instructive, as for the duration experiments, to determine the effect of frequency on the timing of peak expression.

Although the fluid shear stress experiments did show a significant change for the 1 Hz cells, further studies are needed to more accurately describe the fluid flow mechanics of this system. Initially, this should involve identical experiments at higher frequency levels (*i.e.* up to 20 Hz), so that the entire physiologic range may be included. This is important, since the relative influence of fluid shear stress may decrease at higher frequency levels. Alternatively, experiments using a simpler mechanical device, such as a rocking table, which produces variable fluid shear stress in the absence of mechanical deformation, might help to clarify the effects of fluid flow alone. Lastly, the development of a computer model for this membrane stretch system, including graphic as well as quantitative analyses of cellular deformation/fluid shear stress interactions, may be helpful in further clarifying the possible links between these activation phenomena.

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